Pharmacological Evaluation of Shogaol on High Fat Diet and Metals Induced Hippocampal Memory Impairment

Master of Science in Healthcare Biotechnology

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

In

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I certify that this research work titled **"Pharmacological Evaluation of Shogaol on High Fat Diet and Metals Induced Hippocampal Memory Impairment"** is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources has been properly acknowledged / referred.

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Armeen Hameed

Master of Science in Healthcare Biotechnology

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Armeen Hameed

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LIST OF ACRONYMS

As	Arsenic
Al	Aluminium
Pb	Lead
CNS	Central nervous system
HFD	High Fat Diet
hr	Hour
S	Seconds
Kg	Kilogram
M	Molar
µl	Microliter
mg	Milligram
µg	Microgram

ABSTRACT

Both humans and animals are exposed to various metals either simultaneously or sequentially via varying occupational and environmental routes. Recent industrialization has spiked an increase in the environmental exposure to heavy metals, especially Aluminum, Arsenic and Lead, whereas our socioeconomic trends have led to an increased exposure to high fat diet. According to a report by WHO, in 2013, over the previous three decades, obesity has doubled worldwide, becoming a pandemic. Prolonged exposure to HFD and metals can lead to severe neurological impairment and may also cause inflammation in the brain. The present study elucidates the neuroprotective effect of Shogaol, a plant extract from ginger, on HFD and Metal induced neurotoxicity, in mice models. 40 male Balb/c mice were allocated into four different groups (n=10, each group): Group 1 (Control), Group 2 (metals+HFD), Group 3 (metals+HFD+Shogaol 2mg/kg), and Group 4 (metals+HFD+Shogaol 12mg/kg). Different behavioral analysis were performed to determine the learning and memory impairment, which included the following tests: Morris water maze test, Y-maze Test and Hole Board tests, followed by biochemical analysing to check effects of protective effect of Shogaol on liver and kidneys. The results depict Shogaol having a possible neuroprotective role against neurological impairments. Further research needs to be conducted in order to determine the optimum dosage level of Shogaol as a possible treatment for neurotoxicity.

Chapter 1

INTRODUCTION

Learning and memory are, inarguably, two closely linked concepts. While, learning is considered as acquiring skills and/or knowledge, memory is the manifestation of what has been acquired during the process of "learning". The inability to acquire new skills or carry out already learnt task, due to any of the various reasons is called Learning and Memory Impairment.

1.1 Hippocampus and its association with memory formation

Identified as a central part for declarative memory through various studies, hippocampus has been considered vital for learning and memory (Kempermann, 2002). Critical for memory formation and spatial navigation, hippocampus is an integral part of the limbic system. Lesions found within the hippocampal region lead to distressing impairment in declarative memory in both humans, and non-human primates (Luo et al., 2009). An initial report by Scoville and Milner (1957), observed, memory loss in humans after the removal of hippocampus, indicating the involvement of this region in memory formation. The role of hippocampus has been observed in memory formation and it was also determined that memory formation was also selective to both domains of time and space for memory processing (Eichenbaum, 2000).

The hippocampus is considered to be the structure of brain, critically involved in the creation, organization, and recoup memories. The main type of neuronal cells found in the hippocampus region, are excitatory pyramidal neurons (Fig 2). These neurons assimilate information from the surrounding regions which may be, either; spatial

cues, contextual information, and/ or emotional information, and then transfer all the information from the hippocampus to various regions of the brain (Xu et al., 2012).

Both, CA1 and subiculum regions of the hippocampus transmit information via the pyramidal cells, by firing either individual action potentials, or in high frequency bursts. Such unique neuronal firing patterns are important functionally as they may aid in increased reliability of synaptic communication through increased probability of inducing a post synaptic spike (Lisman, 1997) (Williams and Stuarty, 1999). These firing patterns are also involved in evoking plasticity along with the development of place fields (Epsztein et al., 2010); (Golding et al., 2002).

High frequency bursts have been seen to be involved and perform an important role in establishing hippocampal-dependent memories (Xu et al., 2012). Regardless of the functional importance of these varying neuronal firing patterns, it is yet to be determined whether the observed heterogeneity in hippocampal pyramidal cell firing patterns is a reflection of the existence of multiple cell types or a single cell type with varying excitability (Greene and Totterdell, 1997); (Jarsky et al., 2008); Van Welie, et al. 2006).

The excitatory neurons are the pyramidal cells in CA3 and CA1, and the granule cells in the dendate gyrus (Hasselmo et al., 2002). Pyramidal neurons found in the CA regions of the hippocampus have different functional characteristics. The cells found in the CA3 region are activated only via specific sensory inputs and so they are found in precise, specific spatial locations and transfer signals in a single direction (Hasselmo, 2005), CA1 pyramidal neurons act similar to CA3 neurons, in a lesser heightened way (Gasparini et al., 2004). It has been demonstrated through various neuropsychological studies of human amnesia, that any physical damage to the hippo-

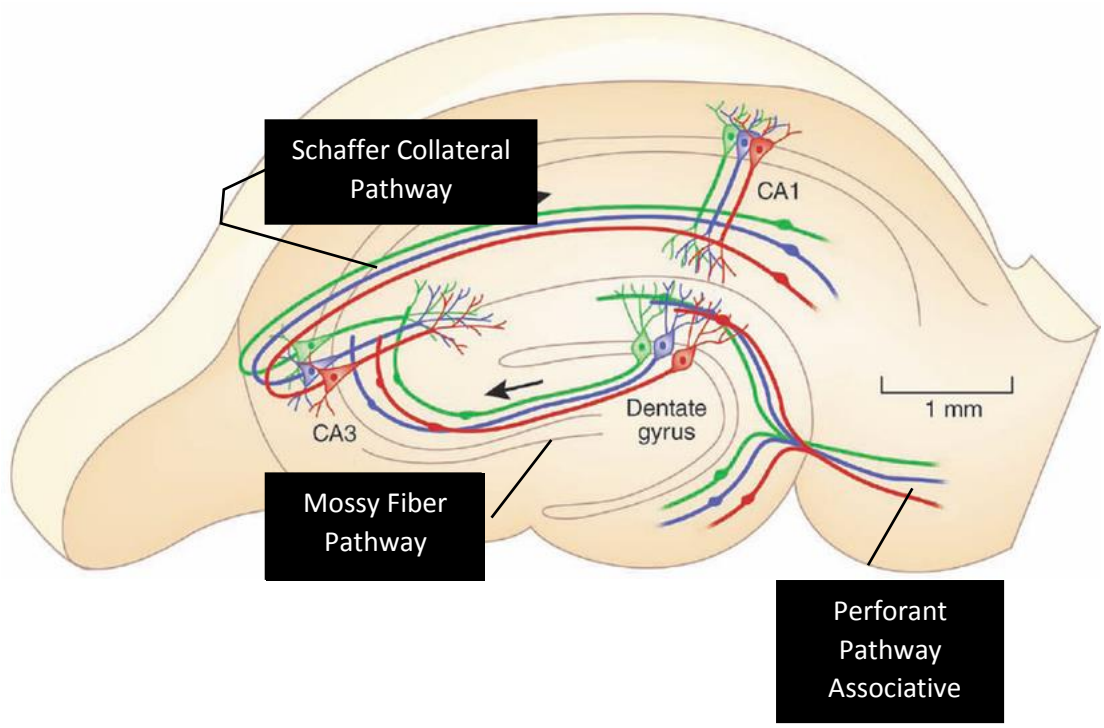

Figure 1.1: Structure of Hippocampus depicting main areas; CA1, CA3 and dentate gyrus. Adapted from (De Michele, 2015)

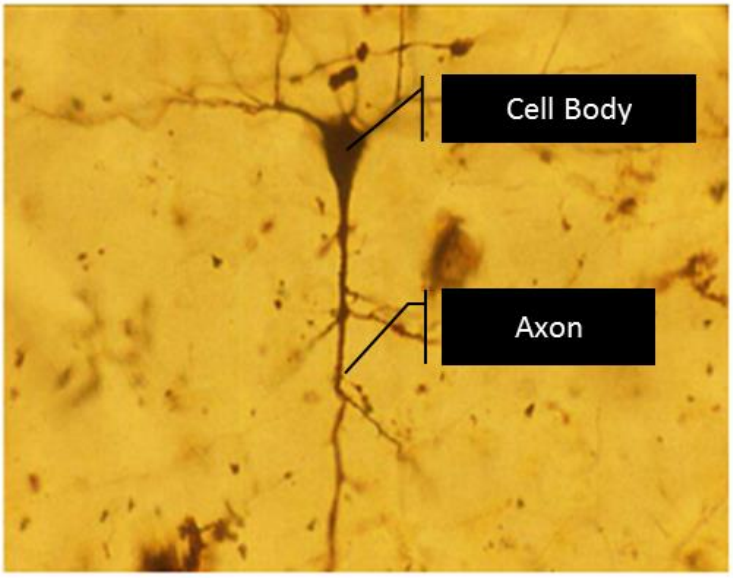

Figure 1.2: Image of pyramidal neuron, found in CA1 cortical region (Silver staining).

-campus, or even a decrease in cell count in the region, results in memory impairment, recollection of memory and the in ability for the expression of explicit memory (Corkin, 1984); (Zola-Morgan et al., 1986).

1.2 Toxicity of heavy metals

The term "Heavy metals" is a vague term, which involves a variety of metals and semi-metals, categorized according to their density. These metals accumulated in the body, causing various disorders. Such hazardous metals include lead, arsenic, cadmium, mercury, aluminium, and iron etc. these metals enter in the body through various pathways and accumulate there, these routes include inhalation of contaminated air, or ingestion of contaminated food and/or water. A global increase in industrialization over the last few decades, has led to increased heavy metal exposure and as a result, has also resulted in declining human health conditions (Rusyniak et al., 2010).

Due to build-up of heavy metals in the body, few essential nutrients/elements start to get substituted for example, lead substitutes calcium, while cadmium substitutes zinc and aluminium substitute majority of the trace elements. The accumulation of heavy metals also leads to many metabolic dysfunctions and also hampers with enzymatic activities and affects the hormonal balance in the body (Mukke and Chinte, 2012). Heavy metal accumulation also results in alterations in the metabolism of carbohydrates, protein and lipids which results in increased susceptibility towards infections (El Safty et al., 2009). Due to alterations in the aforementioned mechanisms, the synthesis of various neurotransmitters is eventually effected along with their use in the body, hence changing the functioning of the central nervous systems (Hyder et al., 2013).

It has been clearly indicated in literature that exposure to heavy metals and alteration in molecular pathways, ultimately results in the production of reactive oxygen species, inducing oxidative stress in the body which leads to various diseases such as cancer, different neurological disorders, kidney dysfunction etc (Mudgal et al., 2010).

1.3 Exposure to metals and associated problems

The maximum permissible limit (MPL) or reference dosage for every metal has been calculated on the basis of data collected via various experiments, are defined as the highest dosage of any chemical at which no health effect has been reported after long time exposure (Jadhav et al., 2007). MPL standards have been devised by the World Health Organization (WHO) for many contaminants that are present or maybe found in drinking water, and so are hazardous for human health. Effects of heavy metals, on the biological system, have been studied in different species and a significant amount of data from exposed animals and humans has been collected. However, chronic low level exposure to toxic metals is still prevalent and continues to pose as a threat, globally (Jadhav et al., 2007).

1.3.1 Aluminium

8% of the Earth's total crust, accounts for Aluminum, which is considered to be a considerable threat to the environment and a major contaminant (Exley, 2012). The biological availability of Aluminium has increased overtime due to various causes, such as increase in industrialized societies, increased use of fossil fuels, etc. (Exley, 2013). Ingestion of Aluminium via food is a main route for Aluminium exposure as its salts are added to food prepared commercially in a variety of ways, such as in food colorings, as emulsifiers, and anticaking agents etc. Aluminium is also added as a clarifying agent to some bottled water in water supplies (Walton, 2014).

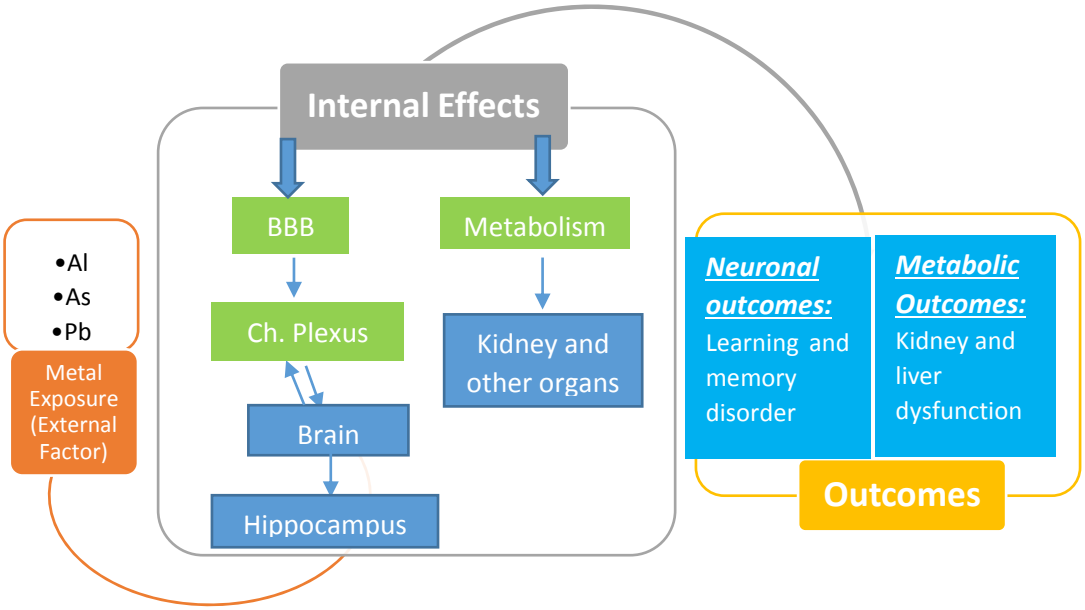

Figure 1.3: Diagrammatic explanation of the effects of metal mixture exposure on the brain. Adapted from (Karri et al., 2016)

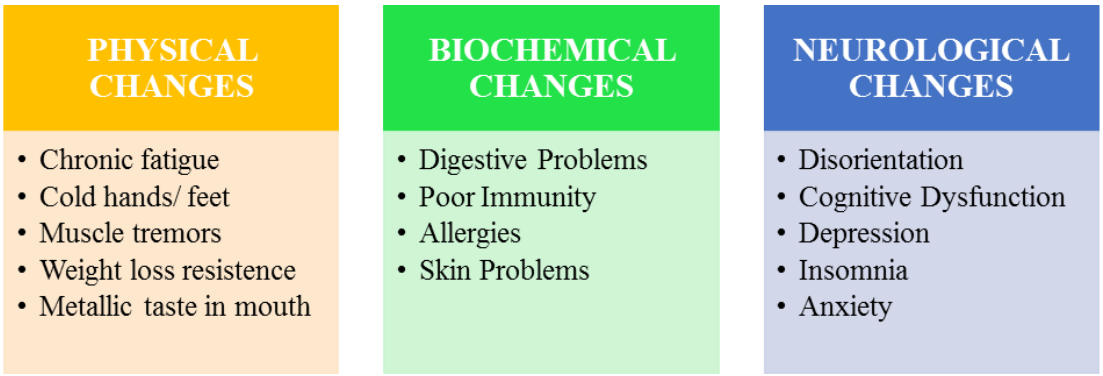

Figure 1.4: Heavy metal toxicity manifestation. The figure depicts the list of biochemical, physical and chemical changes occurring in the body, due to metal exposure.

Salts of Aluminium are used as adjuvants in vaccines, to stimulate immune activation. It is also used some tropical applications, for example sunscreens and deodorants, all accounting for non-dietary Aluminium exposure (Martinez et al., 2017).

While Aluminium shows acute toxicity, data is limited on chronic exposure of Aluminium and its effect on the body. However, it has been indicated that Aluminium might hamper with the absorption of phosphorous which results in weakness, anorexia and bone pain. Tests for carcinogenicity, mutagenicity and teratogenicity (Baker and Schofield, 1982). (Baker and Schofield, 1982)) discovered that the OH and F complexes of Aluminium are extremely labile (inorganic) and may be more hazardous than either organic or particulate forms of Aluminium. Based on the studies conducted by (Driscoll, 1984)), on Aluminium toxicity, established that positively charged species of Aluminium Hydroxy are a lot more toxic for fish than the organic complexes formed of aluminium. Nonetheless the data present on human studies is not sufficient to determine whether which species of Aluminium complexes are more toxic to humans.

1.3.2 Arsenic

Abundantly found on Earth's surface, Arsenic, a metal that is found in air, water and soil; released into the environment through human activities such as excavation, smelting, pesticides etc. (Rodrıguez et al., 2001). Toxicity caused by Arsenic, has resulted in a global health issue, affecting people on a large scale. Exposure to Arsenic is usually due to occupational, accidental or environmental exposure; which results in various diseases for example, respiratory, neurological, endocrine, cardiovascular etc. ((Ratnaike, 2003); (Duker et al., 2005)).

Neurobehavioral dysfunctions have been determined via various epidemiological and animal studies caused by chronic exposure to arsenic. In children, it has been observed that prolonged exposure to high dose of Arsenic, leads to impairment of speech and long term memory (Calderon et al., 2001) memory retrieval and formation patterns, maintaining attention (Tsai et al., 2003), and intellectual function (Wasserman et al., 2004). Rodriguez V, (2002), conducted a study with his colleagues and determined the exposure to sodium arsenite results in changes in learning tasks and alterations in neurobehavioral aspects (Rodrıguez et al., 2002). Comparable results were seen in rodent models, obtained by Martinez-Finley et al., (2011), and colleagues, who determined behavioural deficits that were hippocampus dependent, caused by exposure to arsenic, which induced neurotoxicity. (Bellinger, 2013)), showed neurological deficits in children due to alterations in growth and development caused by exposure to Arsenic at higher concentrations. (Gong and O'bryant, 2010)), determined through various studies, relationship between Alzheimer's disease and exposure to Arsenic. Studies conducted *in vivo*, determined Arsenic exposure's impacted the neuronal synaptic activity, found to be localized in the hippocampus (Krüger et al., 2006).

Arsenic toxicity also presents an autoimmune disorder that is often mistaken for Guillain-Barre syndrome. In this case, the body's immune system starts to attack parts of the Peripheral Nervous System (PNS), which results in the inflammation of nerves ultimately causing muscle weakness ((Kantor, 2006); NINDS 2007).

1.3.3 Lead

The most commonly found heavy metal, lead, adds up to a total of 13 mg/kg of the Earth's crust. Exposure of lead over a life time, has varied effects on the

neurophysiological functions. Previous studies conducted have shown increased prenatal exposure to lead results in underdeveloped sensory and visuo-motor areas of the fetus' brain ((Bellinger et al., 1987); (McMichael et al., 1988)). Exposure to lead affects the entire body, mainly the hematologic, renal and neurological systems. Lead toxicity leads to an increased amount of erythrocyte destruction and also inhibits the synthesis of heme. Hence, most of the physician account of lead exposure as a possible cause for microcytic anemia (Atlanta, 2002). A neurotoxin that has effects on almost every organ of the body, in children, lead has the ability to slow down cognitive development along with affecting their intellectual performance and induce damage to kidneys and also the reproductive system (Qin and Chen, 2007).

There are multiple routes for lead exposure some which include; accidental soil ingestion, consumption of lead contaminated food, inhalation of contaminated air, however inhalation of contaminated air accounts for the least possible exposure (Davies et al., 1990), while consumption of contaminated food is the most common route of exposure and cause of lead neurotoxicity ((Chen et al., 2016); (Lanphear and Roghmann, 1997)). vegetables being an vital source for nutrition in humans, needs to be assessed critically for lead pollution in order to determine their safety for consumption (Goswami et al., 2012)

Effects of Lead toxicity in the body

Being a toxic substance, lead has numerous detrimental effects on the human body; discussed below:

a) Intelligence, Memory and Language

A study conducted by WHO, determined a reduced intellectual functioning if human blood showed lead levels less than 25 µg/dL. Study revealed that a drop in

Intelligence Quotient (IQ) from 1-5 points have been observed with each 10 µg/dL increase in lead concentration in the blood. Results of another research conducted by Khalil et al. (2009) observed a decline in cognitive functions as a result of increased exposure to lead. Many researchers have determined that due to increased lead exposure, various abilities like, memorizing the visual cues and visuo-spatial abilities are hindered.

Studies have shown that continuous exposure to lead, leads to a continuous decrease in both memories; non-verbal and verbal. In a finding, the people that were given lead exposure of up to 20 µg/dL, manifested a decrease in executive functional capabilities. It was concluded in a research carried out by Strolley et al. (1991) that lead concentration in the blood when up to or higher than 40 µg/dL, results in decreased ability of the individuals to make decisions, along with a slower speed for classifying things. Previously conducted studies have concluded that lead exposure effects an individual's reading capability. A study on older man has concluded that long term exposure has effects on human language. The ability of an individual to define words and name drawings via visualization is also greatly affected due to lead exposure.

b) Neurotoxic effects

Regardless of the various was that lead may be absorbed by and accumulated in the body, once inside, it has two main effects on the nervous system.

1. Metabolic effects

Lead causes morphological changes some of which are the major players in affecting the nervous system. These morphological changes are as follows:

i. Decreased production of sialic acid in neurons, thus interfering with the formation of synapse (Bressler and Goldstein, 1991)
ii. Damage to some important molecules during migration and differentiation (Silbergeld et al., 1992)
iii. Glial cells differentiation in early stages (Cookman et al., 1993)

2. Pharmacological effects

Pharmacological effects of the metal, lead include its ability to replace calcium and zinc ions in important pathways. Lead accumulation causes alterations in release of neurotransmitters which results in the altered functioning of three main systems found in the brain that are namely: Cholinergic systems, dopaminergic, and GABA-ergic systems. Other pharmacological effects during neonatal course include inhibition of NMDA ion channels (Guilarte et al., 1993).

c) Hemolytic anemia

Synthesis of heme involves three main enzymes namely;

1. Aminolevulinic acid synthetase (ALAS)
2. Aminolevulinic dehydratase (ALAD)
3. Ferrochelatase

Many studies have concluded that, long term and repetitive exposure to lead, blocks the activity of these enzymes, hence hindering the synthesis of heme. ALAD, being the most affected by lead exposure, is commonly implied as a common biomarker for evaluating lead induced toxicity.

d) Cardiovascular effects

According to researchers, increased exposure to lead and its levels in the total blood has a correlation with an elevated risk of cardiovascular diseases. According to a study conducted by Sirivarasai et al., 2015, it was found that the mechanism linking deaths due to lead exposure and cardiovascular diseases might have some involvement of oxidative stress caused in the body due to lead toxicity.

e) Reproductive toxicity in humans

Lead has famously been linked with male and female infertility. In females, increased lead levels in blood has been linked to increased number of miscarriages and still births, while in males, increased lead levels have been linked to a decreased total sperm count, as well as decreased libido and vigor (Levin and Goldberg, 2000).

f) Vitamin D deficiency

Vitamin D deficiency results due to increased exposure to lead as the metal directly effects the pathway responsible for the conversion of vitamin D into 1,25-dyhydroxyvitamin D; as determined by Kemp et al. 2007.

g) Elevated risk of cancer

Lead, a known carcinogen, cause cancers such as cancers of bladder, stomach and lungs. Lead has been classified as a carcinogenic agent for humans, after collecting evidence from several animal testing and some human studies (IARC, 2004).

h) Lead exposure and pregnancy

A study conducted by Potula and Kaye, (2005) revealed several after effects of lead exposure during pregnancy. Some of these effects included the following:

- Premature births,
- Low body weight of fetus at the time of birth,
- Growth impairment of fetal bones (this is caused because lead competes with calcium for deposition in bones).

Studies conducted previously have determined that children, who are exposed to lead, show varied brain volume (Cecil et al., 2008). The CDC (Centre for Disease Control) has limited lead exposure in children i.e. up to 10 μg/dl in blood (Landrigan, 2000). This defined limit is estimated to be the upper limit for causing possible cognitive deficiency in children (Jusko et al., 2008). Early post-natal exposure to lead has shown to create a greater learning impairment in animals as compared to animals, exposed to lead in older ages (Kuhlmann et al., 1997).

Lead contamination is a worldwide problem. Studies conducted in India determined high concentrations of lead found in drinking waters of Assam (Borah et al., 2010). Studies conducted in Bangladesh confirmed concentrations of lead surpassing the defined permissible limits, in drinking water of tube wells (Frisbie et al., 2008). Studies conducted in areas of Africa determined contamination of water with lead and other heavy metals.(Asante et al., 2007). Likewise, in an examination led by Wyatt et al, 1998, in Northern part of Mexico determined lead concentrations varying between 50 - 120 μg/L. Furthermore, a research conducted in Lake Burragorang (Australia) showed the concentration of lead to be 332 μg/g (Birch et al., 2001). Similarly, researchers in China, concluded lead to be a major toxic contaminant in the rivers of China (Wong et al., 2003). After reviewing all related literature, WHO concluded, in a program that if lead levels in the blood exceeds 25 μg/dL, it reduces the intelligence quotient (IQ) significantly. Moreover, studies confirmed that with every 10 μg/dL elevation in blood lead levels, a decreased IQ is observed between 1 to 5 points.

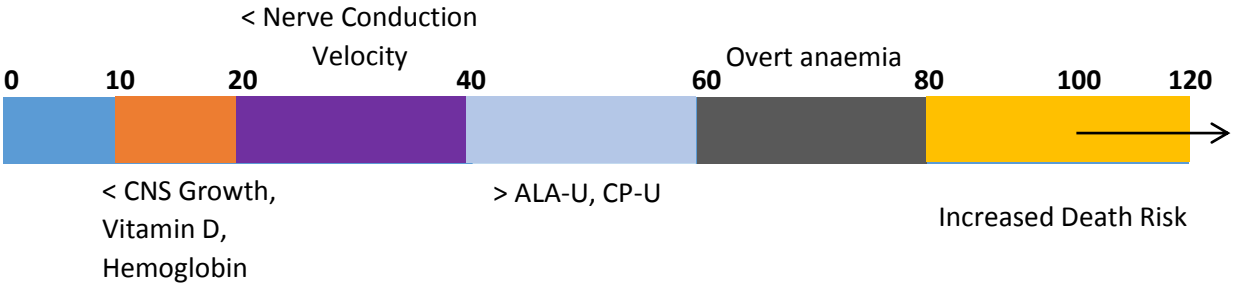

Figure 1.5: Lead exposure and its effects on health (µg/dL)

In a research performed by Khalil and his research group, concluded effects of increasing exposure of lead on low levels of cognitive functions. This study also showed effects of varying lead effects on intelligence (Khalil et al., 2009). It has been concluded by many researchers that exposure to lead effects an individual's visuo-spatial ability, to learn and recall the visual stimuli. Sufficient studies have also concluded that continuous exposure to lead, as a result leads in a decline in non-verbal and verbal memory. Researchers have concluded in a study that a maximum exposure of lead, up to 20 μg/g, results in reduced executive functional output.

In a research put forward by Stollery and his group, it was concluded the lead levels exceeding the blood level of 40 μg/dL, for any individual, results in decreased decision making abilities, and the speed at which an individual classifies things. (Stollery et al., 1991). In both, adults and children, the focal reason for lead associated toxicity is the central nervous system. In the case of adolescents their developing biological systems are the main areas for lead related toxicity, effects of which can be observed at blood levels.

By interacting with the NMDA receptors, lead mainly disturbs the hippocampus region of the brain (Fig. 6). As reported by Guilarte and Miceli (1992), interaction between lead and the NMDA receptor is both voltage dependent and also non-competitive (Guilarte and Miceli, 1992). Calcium ion signalling mechanism is mainly disrupted by lead, in neuronal synapsis via the NMDA-Glutamate process. The subunits of NMDA receptors': NR2A and NR2B, expression are modified by interaction with lead, which also forms complexes, leading to the deregulation of calcium associated sensitive signalling pathways in the hippocampus region of the brain. (Toscano and Guilarte, 2005).

As determined by Zhang et al. (2002), prolonged lead exposure results in decreased NR2A content while the expression of NR2B increases, in the hippocampus. Similar outcomes were also supported in a research conducted by Neal et al. (2011), who confirmed via cell culture study that lead down regulates and decreases the expression of NR2A subunit of the NMDA receptors. Kim et al. (2005) determined that both NR2A and NR2B receptors are important for the expression of NMDA receptors and the neuronal activity.

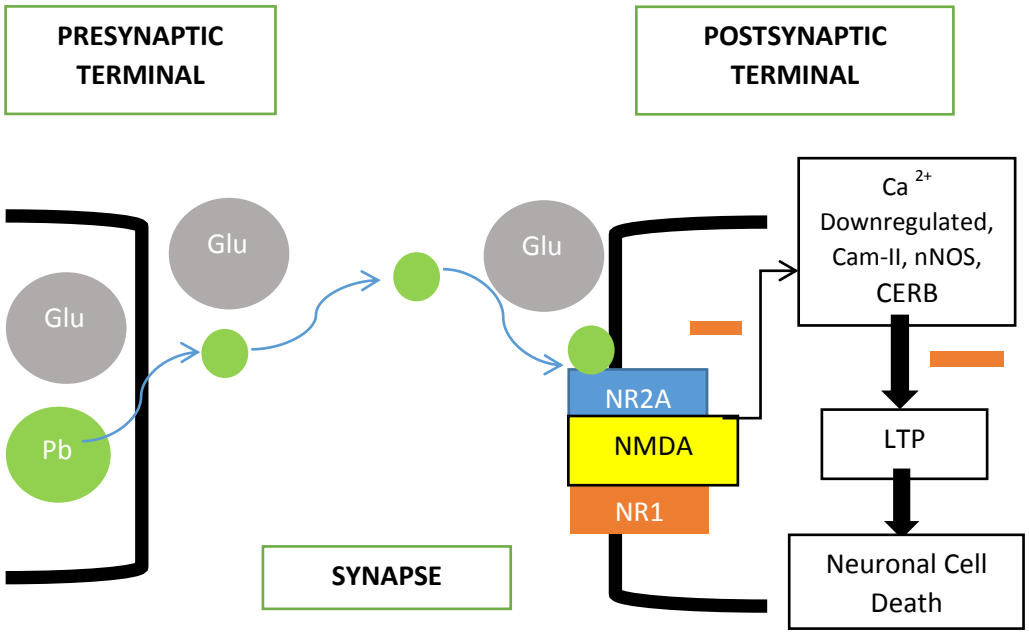

Figure 1.6: Schematic Representation of entry of lead ion into hippocampal synaptic region. Lead enters by competitive mechanism with glutamate and high affinity to binding with NMDA receptor, forming Pb-NMDA complex.

Glu= Glutamate

Pb= Lead

LTP= Long term Potentiation

Table 1.1: Routes and health impacts of exposure to heavy metals

Name of Metal	Route of exposure	Major Sources	Health impacts
Arsenic	Consumption via drinking water, food, smoking, occupational	Smelting, fossil fuel burning, agricultural pesticides, industrial waste ((Hughes et al., 2011); (Chilvers and Peterson, 1987)	Arsenicosis, psychological effects, (Brinkel et al., 2009); (Keya, 2004), decreased mental performance (Chen et al., 2004), hypertension, cardiovascular disease risk, carotid atherosclerosis and diabetes mellitus, (Chen et al., 2006, Wang et al., 2007, Chen et al., 2009)) lung cancer (Hubaux et al., 2013) carcinogenesis (Salnikow and Zhitkovich, 2007).
Lead	Water ingestion, paint, soil	Mining, fossil fuel burning, manufacturing of lead—acid batteries, oxide synthesis for paint, and pigments ((Gabby, 2006); (Jacobs et al., 2002)	Neurotoxic effects on intelligence (Khalil et al., 2009) decreased memory (Schwartz et al., 2000), hemolytic anaemia (Vij and Dhundasi, 2009), CVD diseases[1] (Navas-Acien et al., 2006), reproductive toxicity (Levin and Goldberg, 2000),

[1] CVD: Cardiovascular disease

			lung cancer, bladder cancer (IARC, 2004)
Aluminum	Salts of Aluminium are used commercially to prepare foods for various reasons, for e.g. food coloring. Non-dietary sources of Aluminium in pharmaceuticals	Cosmetics, antiperspirants, beverages cans and foils, antacids and water treatment plants (Keith et al., 2008).	Ischemia (Costello et al., 2014), kidney diseases (Kurella et al., 2004), pulmonary abnormalities (Mazzoli-Rocha et al., 2010).

1.4 Pakistan and its water contamination

Water contamination has been a leading cause for concern in Pakistan for decades. Metal exposure is causing serious health issues around the country for all those who are exposed to this contaminated water. The metabolic capabilities of the body can be disrupted in many ways via heavy metals. Furthermore, metals can accumulate in heart, liver, kidneys and brain, all of which are the vital organs of the body, there they can disrupt standard biological functions. When heavy metals are inside the organic frameworks they obstruct their imperative exercises in body (Rehman et al., 2018).

Heavy metals like lead and arsenic have been reported in underground and surface water resources throughout the country in Pakistan (Fig. 7). Harvests of this sullied water are also found to have these metals' poisonous concentrations. To help the advancement of manmade innovations, metals were found and extricated to be utilized as an instrument to satisfy the necessities of people. In any case, presently, people along these lines are headed toward damaging health impacts due to the contamination of water and soil surfaces with these metals. Living frameworks don't cut down heavy metals, which at last prompts their gathering up to destructive levels (Beyersmann and Hartwig, 2008).

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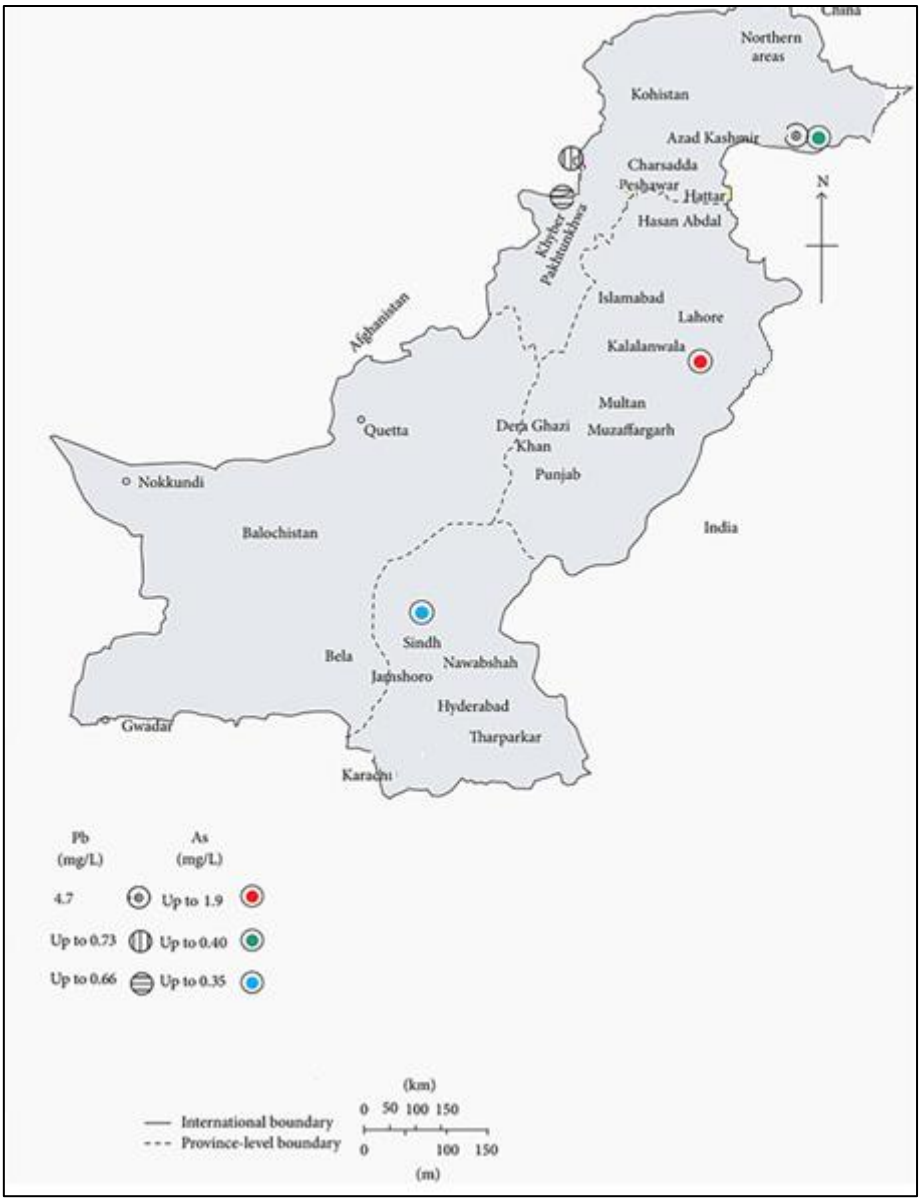

Figure 1.7: Pakistan's map depicting the concentrations of lead, and arsenic in ground water**.** Adapted from (Rehman et al., 2018)

The most extreme allowable drinking water content of Aluminium is 200 g/L (WHO, 2004). These days, much intrigue is raised on the poisonous quality and harmful health effects that Aluminium might have (Venturini-Soriano and Berthon, 1998). A few examinations recommend that Al might be collected in the brain by means of various courses (medicine, drinking water and food) and meddle with the normal exercises of the nervous system (Flaten, 2001; Sińczuk-Walczak et al., 2005). This particle of metal is considered as a conceivable reason for Parkinson's and Alzheimer's diseases along with renal osteodystrophy (Flaten, 2001). The assurance of exceptionally low degrees of Aluminium has turned out to be progressively significant in ecological and clinical science because of the negative role it plays in human life (Bishop et al., 1997).

An investigation directed in Pakistan, by Ahmed et al., (2004) and Saqib et al., (2013), uncovered that two Punjab and Sindh, the main leading provinces, brought about higher concentrations of Arsenic. Both Punjab and Sindh were reported to have water resources with arsenic concentration more than 50µg/L. After an overview in 2001, 9% of tests out of 8712 examples showed that 35 areas of Pakistan have As concentration more than 10µg/L while 0.70% of tests showed it to be above 50µg/L (NAPAM, 2007-2011).

One of the As advanced zones recognized was Muzaffargarh because of an overview led by Pakistan's public health engineering department along with the UN's International Children's Fund (UNICEF) in 2001 (Shrestha, 2002). An overview led in 2007, determined four towns in Punjab with higher As concentration being 681µg/L in Waran Piran Wala, 672µg/L in Manga Mandi, 883µg/L in Shamkey Bhattian and 2400µg/L in Kot Asad Ullah and Kalalanwala (Farooqi et. al., 2007). In like manner, the concentration of As in Southern Punjab is additionally worrisome as

it is causing medical issues in view of that it is utilized for drinking water and irrigation purposes. Another study came about concentration of Arsenic extending between 35.2-158 μg/L in Manchar Lake (Southern Sindh) (Arain et al., 2009; Arain et al., 2009)

The recommended drinking water concentration of arsenic is 10 μg/L (WHO, 2017). All over Pakistan, many overviews have demonstrated that concentrations of arsenic in groundwater > 10 μg/L are normal, with a little % surpassing the provisional value for Pakistan of 50 μg/L (Pakistan Environmental Protection Agency, 2008): such concentrations represent a hazard to human wellbeing (Ahmad et al., 2004). By 2004, an aggregate of 36% of the number of inhabitants in Province Sindh, and 20% of the number of inhabitants in Province Punjab, were evaluated to be in danger from arsenic in groundwater (Ahmad et al., 2004). Utilizing populace figures from the 2017 census (Pakistan Bureau of Statistics, 2017) and expecting 80% of the populace drink groundwater, these figures extrapolate to around 11 million in Punjab and 4 million in Sindh in danger.

In Pakistan, the concentration of lead was observed to run from < 0.001-4.7 mg/L in different regions in most of the water samples and this concentration surpasses the allowable limit for drinking water set by WHO (0.01mg/L). Some of the water samples collected from AJK Pearl valley showed lead concentration extending somewhere in the range of 1.8 and 4.7 mg/L (Javaid et al., 2008). In like manner, the samples of water from KPK's Hattar Industrial Estate, indicated lead concentration to be almost near 0.26mg/L which is also above the allowed limit (Manzoor et al., 2006).

Over half the investigations directed to check waste water lead levels inferred lead levels to be higher than 0.50 mg/L (the admissible range) of waste water lead, the

range set by National Environmental Quality Standard Pakistan (NEQS) (NEQS, 2000). The elevated amounts of lead in the waste water are perilous for the soil, crops, plants and furthermore for individuals. Additionally, when the lead concentration of soil was determined in Kohistan Region of GB, it identified to be 103,000 mg/kg in contaminated soil (Muhammad et al., 2011). Lead levels of 121 mg/kg were available along urban Karachi in beach front slit of the Arabian Sea (Siddique et al., 2009). At the point when lead levels were tried in shallow residue of River Lyari they were observed to be 49.5 mg/kg (Mashiatullah et al., 2013).

Lead is viewed as a lethal constituent as a result of emission coming from the combustion of oil and coal in industries at the level of 450 million kilograms for each annum while naturally discharged lead levels were observed to be 30 million kilograms for every annum (Kabata et al., 2011). Lead levels were found to exceed the limit 0.03-44 mg/kg in different types of vegetables in Pakistan (Abbas et al., 2010). An investigation directed in Northern Punjab's area Gilgit found that most astounding concentrations of lead were present in Msylvestris (Khan et al., 2010; Khan et al., 2010).

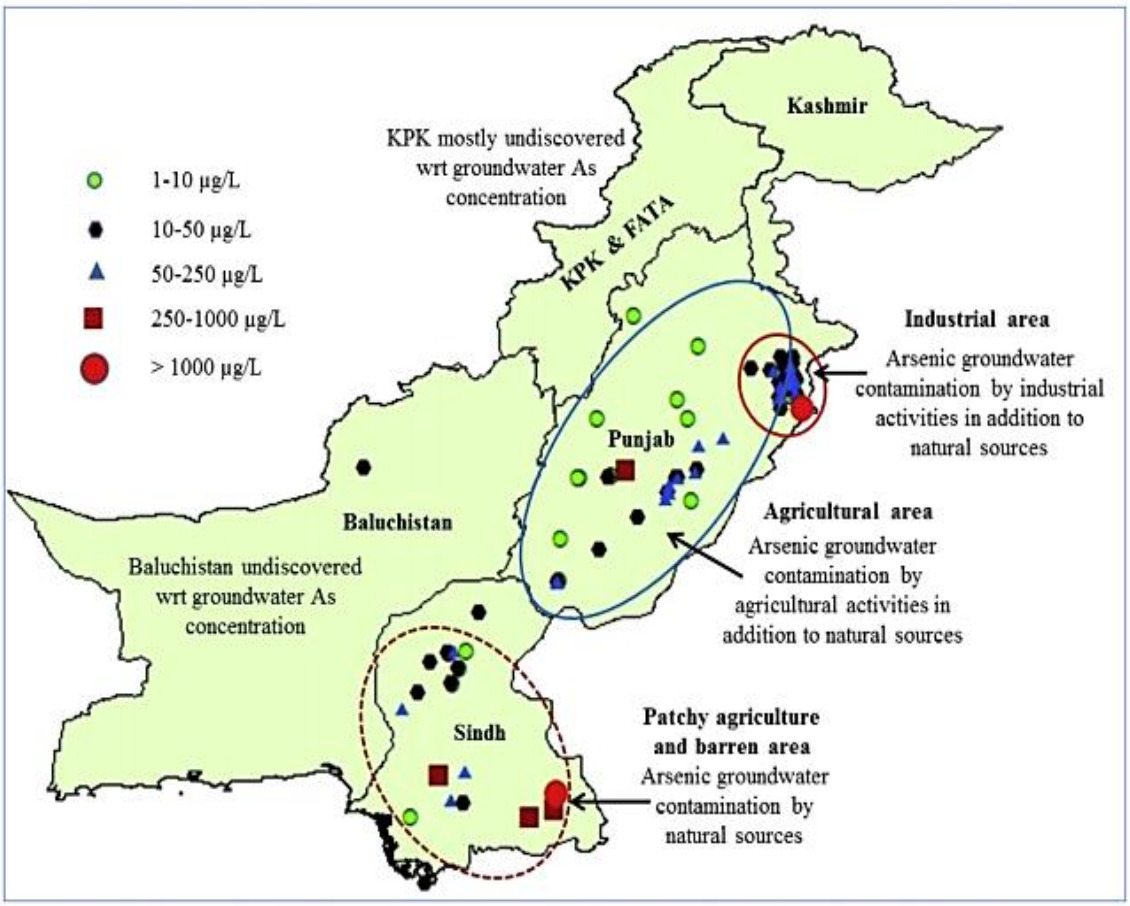

Figure 1.8: Map of Pakistan showing Arsenic (As) concentrations found in different areas of various provinces. (Adapted from Shahid et al., 2018)

Table 1.2: The maximum acceptable values (mg/L) of drinking water quality standard

Metal	**WHO standard value (mg/L)**	**Pak- EPA Standard (mg/L)**
Arsenic	0.01	≤ 0.05
Lead	0.01	≤ 0.05
Aluminium	< 0.02	≤ 0.02

1.5 Obesity due to High Fat Diet

Obesity has become a pandemic, after nearly doubling worldwide in the last three decades (WHO, 2013). Increased intake of food that are energy-dense is considered to be the major cause for the present rise in overweight and obese individuals, including both, children and adults (Ervin and Ogden, 2013). The occurrence of type-2 diabetes has increased at a startling rate due to an alarming increase in the trend of consuming high fat diet and obesity. Moreover, several researches have suggested high fat diet's role CNS development along with declining cognitive performance ((Solfrizzi et al., 2003); (Lindqvist et al., 2006)). Obesity is found to be interesting, in both obese patients and rodents, and is perceived to be an inflammatory disease because both microbiota found in the gut along with the adipose tissue, add to low grade inflammation in chronic peripheral ((Clément et al., 2004);(Cottam et al., 2004)). Increased levels of pro-inflammatory cytokines are also found to be associated with obesity in a rodent's brain. It has been observed by many researchers along with (Dinel et al., 2011), that brain inflammation caused by obesity, has direct relation with impairment of hippocampal dependent memory.

In developed countries, the trend for diet, with increased content of saturated fats along with refined sugar in increasing at an alarming rate, in adults. Recent data indicates that on average, 12% of daily energy intake of an American adults' is attributed towards added sugars (13%) and saturated fats (Micha et al., 2014), and this is considerably higher than the recommended limit (5-10%) defined by the US Department of Health and Human Services and the Department of Agriculture. A study conducted by Fryar et al., (2016), depicted a prevalent increase in obesity occurrence rate amongst adults in the US, to a total of 37%, which is a significant increase, compared to the prevalence rate of 13%, in 1960.

A potential rise in reactive oxygen species (ROS) is observed due to increased intake of high fat diet. This results in oxidative stress by increasing ROS which further leads to obesity via oxidation of low-density lipoprotein (Xu et al., 2009). Many studies have recently shown the potential role antioxidants along with anti-inflammatory compounds play in suppressing obesity. A research carried out by Holloway et al., (2011), in adults, showed a significant attention deficit and reduced speed of retrieving information from working and episodic memories, who had taken HFD for 5 days, compared to those adults on a standard diet. A lot of studies have suggested the role of HFD in affecting the hippocampus and compromising its function and structure by its immune cells sensitization, leading to inflammatory response priming (Sarah et al., 2017).

Research has shown, that in the hippocampus, increase in the levels of pro-inflammatory cytokines results in the deterioration of several, varied mechanisms involved in generating synaptic plasticity and eventually effecting the long term memory (Barrientos et al., 2015). In a study conducted by Sobesky et al., (2016), the conclusion was, that by the intake of HFD, the hippocampal cells are primed and by increasing the levels of glucosteroid hormone (corticosteroid) in the region.

1.6 Obesity becoming an epidemic in Pakistan

Based on a study carried out in 2006, around 25% of the Pakistani population is characterized as obese or overweight (Jafer et al., 2006). This figure was computed relative to the Indo-Asian BMI thresholds. Since then, the Pakistani population has been drifting towards non communicable and preventable diseases including obesity (Jafar et al., 2013). 2016 WHO statistics reveal, that the percentages of population for overweightness and obesity are 20.8% and 4.8% respectively (WHO, 2016).

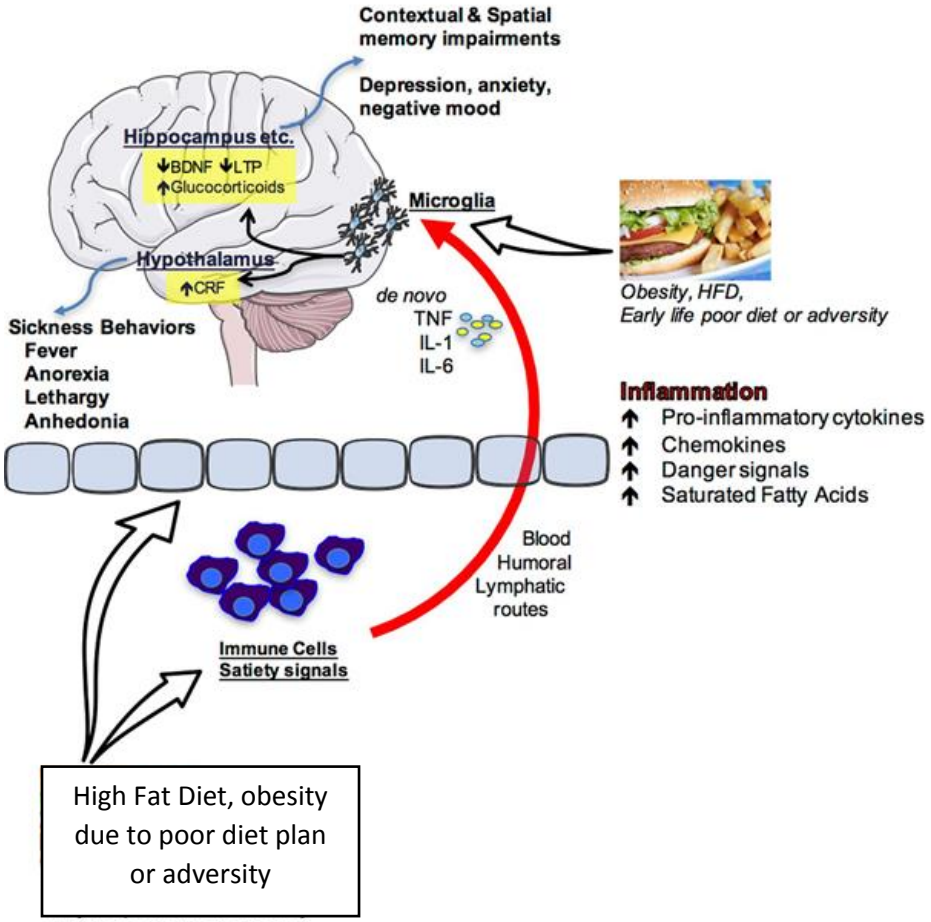

Figure 1.9: Schematic diagram representing the influence of nutrition cognitive and emotional functions. Obesity, overeating or too much consumption of HFD etc., may result in inflammatory response in immune cells of both CNS & PNS, along with affecting the blood–brain barrier (Adapted from: Spencer et al., 2017)

The consistency of these numbers reflects the endemicity of overweightness and obesity to Pakistani society. The Pakistan national health survey (1990-1994) reported gender split in the prevalence of obesity among adults of 25-44 years age belonging to rural regions to be; 14% women and 9 % men. While urban areas depicted much higher prevalence: 37% for women and 22% for men (PMRC, 1998). The Pakistan demographic health survey of 2013 also provided evidence of high obesity prevalence attributed to the females of Pakistan (NIPS, 2013). 11% of men and 19% of women in rural areas, while 23% of men and 40% of women in urban areas were found to be obese in a report published in 2013 (PDHS) (Khan et al., 2004).

One research observed the range of overweightness and obesity in the northern regions of Pakistan. The study concluded prevalence of 13.5% in men and 14.1% in women, with same level of increase for both genders per year (Shah et al., 2004). Another study observed Multan, a city of Pakistan while considering the new recommended Asians BMI thresholds concluded that 46% population were obese or overweight while 24.55% were those who are underweight. The comparison between the mean BMIs of males/females were different significantly; 55.12% for males and 36.15% for females – being in conflict with other findings from studies conducted in other regions and cities of Pakistan (Aziz et al., 2009).

1.7 Pharmacological activity of Ginger and its constituent; Shogaol

Ginger has been used extensively as a spice and also as medicinal herb in traditional herbal medicine. Being a natural dietary root, it exhibits certain anti-inflammatory, anti-carcinogenic and anti-oxidative properties (Mohamed & Al – Okbi, 2005; Aldebari et al., 2013). Ginger comprises of many pungent elements including gingerols, shogaols, paradols, and gingerdiols. The volatile oil components are usually

diverse terpenoids whereas the non-volatile compounds include 5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-3-decanone (gingerol), 1-(4-hydroxy-3-methoxyphenyl0dec-4-en-3-one (shogaol), and some other like zingerone, paradol etc.

Ginger with its components:

1. Exhibit antioxidant properties when preventing the loss of macromolecules the triggered by ROS and oxidative stress.
2. Play an important role in anti-inflammatory responses. Previous reports on ginger preparations in vitro and some gingerol-associated compounds revealed exemplary anti-inflammatory responses for example, inhibition nuclear factor κB and of () (Grzanna et al., 2005; Tjendraputra et al., 2001).
3. Have antitumor effects via some modifications in genetic pathways, for example, by activating tumour suppressor gene, modulating apoptosis and inhibiting VEGF. Studies conducted before have expressed that ginger components; terpenoids, activate p53 which in turn, prompts endometrial cells apoptosis (Liu et al., 2012).
4. Possess antimicrobial properties mainly attributed its components like shogaol, gingerol, paradol, zingerone etc. Another discovery disclosed that ethanolic ginger extract (10%) had the potential for antimicrobial activity against pathogens (Giriraju & Yunus, 2013).

Some diseases for which ginger has proven effective include fever, vomiting, arthritis, ulcer, hypercholesterolemia and migraine (Ali et al., 2008). Some active compounds which can be found in ginger include; shogaol, gingerol, paradol, zingerone etc. (Shukla & Singh, 2007). The pungent and predominant compound of ginger, Shogaol, is dehydration product of Gingerol (Connell & Sutherland, 1969). It is

renown that 6-shogaol has more anti-inflammatory and anti-oxidant properties compared to the 6-gingerol. One extremely vital study depicted that 6-shogaol has neuroprotective effects against transient global ischemia via inhibiting microglia (Ha et al., 2012). To further support the significance of ginger as a neuroprotector, another study suggested that ginger accelerates the anti-oxidant defence system of brain while it down regulates the MDA levels to normal in diabetic rats (Shanmugam et al., 2011). Ginger was also found to increase the levels of SOD, GST, CAT, GPx, QR, GR and certain other proteins while decrease the levels of LPO showing protective effects in treated rats (Sharma & Singh, 2011).

Intriguingly, latest researches have shown 6-shogaol to be biologically more active than 6-gingerol (Weng et al., 2010; Peng et al., 2012; Wu et al., 2010; Dugasani et al., 2010). Bhattarai et al. 2001, have recently shown that in the presence of high temperature and acidic conditions in a model system, 6-gingerol can be debased to 6-shogaol.

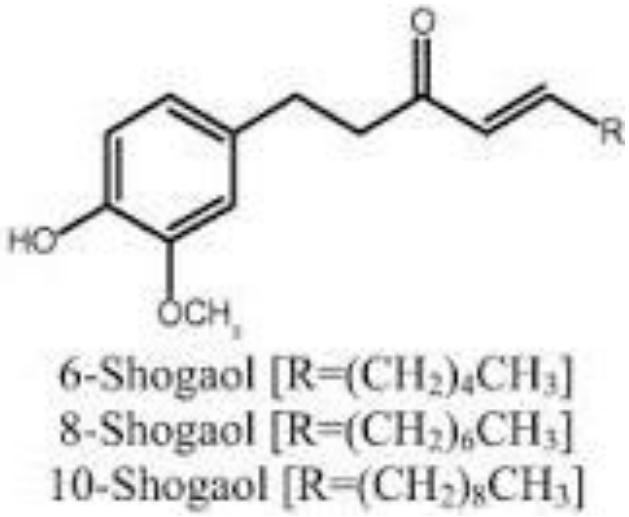

Figure 1.10: Structure of Shogaols

Chapter 2

Materials and Methods

2.1 Ethical Statement and policy

The IRB (Internal Review Board) at ASAB, NUST had reviewed and approved all the protocol performed. The mice were lodged in ASAB's Animal House Laboratory, under controlled conditions. All tests conducted were in accordance with the declarations of the Laboratory Animal Research Institute, Earth and Life Sciences Division, National Institute of Health, USA (Guide for the Care and Use of Laboratory Animals: Eighth Edition, 2011).

2.2 Animals

The research was conducted on male BALB/c mice, provided by NIH, Islamabad and Laboratory Animal House at our institute. The mice were of 3-6 months old and weighed between 25- 30 kg at the time of experiment. All animals were kept in plastic, group cages which measured 45 x 45 x 20 cm, with wood shavings as bedding. Standard housing conditions were maintained for the animals at 22 ± 2 ºC, under 12-Hour light/dark cycle with free access to feed and water.

2.3 Reagents

Shogaol, the ginger extract used in this study was graciously provided to the Neurobiology Laboratory by, Dr. Zaman, Allama Iqbal University, Islamabad.

2.4 Study Design

2.4.1 Animal Groups

A total of 48 healthy lab animals were randomly divided into four equal groups, as given in the table below (Table 1). The details of the groups are as follows:

a) Control Group**:** Animals received deionized water and normal feed.

b) HFD + metal mix Group (Diseased): Animals received 25 mg/kg of each metal (Al, Pb, and As) along with 40% HFD, no treatment.

c) Diseased + Shogaol (2mg/kg): Animals received 25 mg/kg of each metal (Al, Pb, and As) along with 40% HFD. Shogaol was administered from Day 1 of the experiment. 2 mg/kg was mixed with 40% HFD feed and given to the mice.

d) Diseased + Shogaol (12mg/kg): Animals received 25 mg/kg of each metal (Al, Pb, and As) along with 40% HFD. Shogaol was administered from Day 1 of the experiment. 12 mg/kg was mixed with 40% HFD feed and given to the mice.

2.5 Study Timeline

The study duration planned for our work was designed over a period of 60 days, in total. The animals in Group 1 were kept as the "CONTROL" animals, while the remaining 3 groups (Groups 2-4) were subjected to metal mix and HFD exposure from Day 1. Groups 3 and 4 received treatment with Shogaol from Day 1. Tests for behavioural analysis were performed from the 61^{st} to 75^{th} days. Blood was drawn for Biochemical analyzing on the 61^{st} day as well.

Table 2.1: Description of Groups

Group No.	**Description**	**No. of Mice**
1	Control	10
2	Heavy metal + HF diet	10
3	Heavy metal + HFD + Treatment Dose 1	10
4	Heavy metal + HFD + Treatment Dose 2	10
Total No. of Mice		**40**

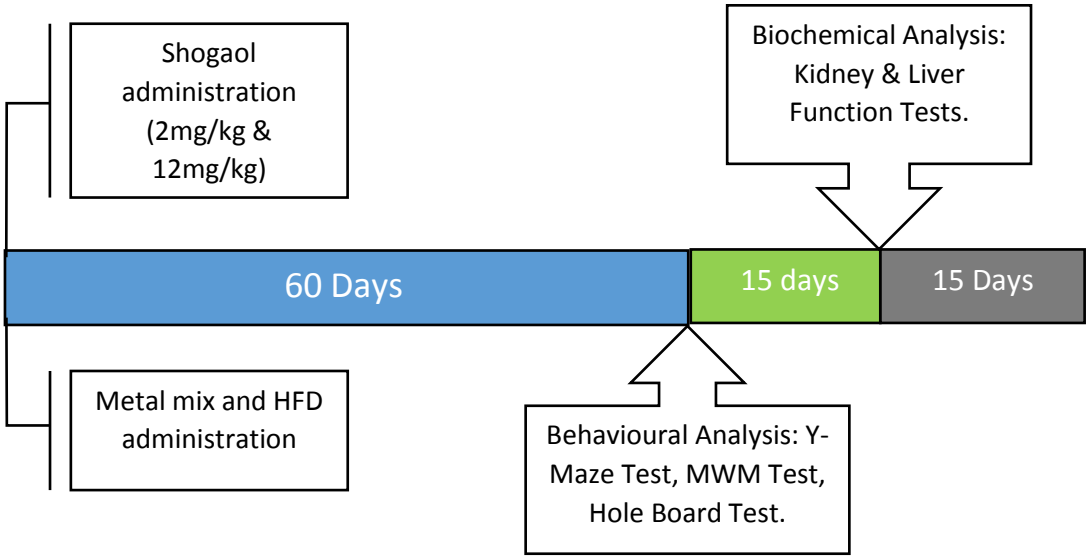

Figure 2.1: Diagram representing the Study design

2.6 Methodology

The following tests and procedures were performed on the animals and results were later analyzed.

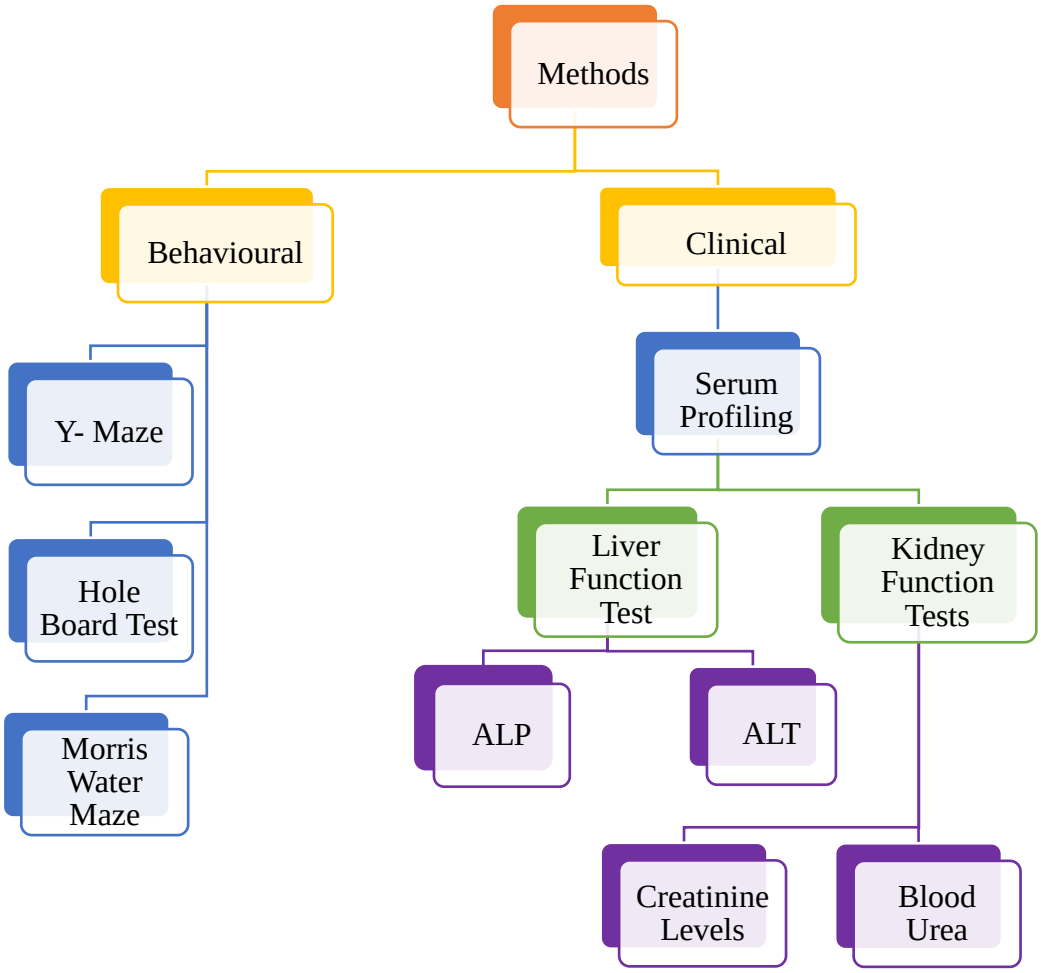

Figure 2.2: Flow chart of all the experiments performed.

2.6.1. Behavior tests

2.6.1.1. Y-Maze

Purpose of Test

This Hippocampus based test measure the spatial learning and memory (both working and recognition), by utilizing a rodent's natural exploratory characteristics. The Y-maze consists of three arms, of equal lengths, interconnected at 120º measuring 50 x 16 x 32 cm to mark the end of each wall of the maze, different patterns in black and white will be used. The test is performed to measure the willingness of rodents to interact with a novel environment. Rodents normally choose to explore a new arm of the maze instead of returning to the one that was previously visited. Various parts of the brain are involved in this procedure; mainly the Hippocampus and pre-frontal cortex.

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

Procedure

The test will be performed by adding a few modifications to the Y-maze protocol, previously the described by Dellu et al., 1992, and Conrad et al., 1996. The three arms of the maze will be labelled as start, other and novel arm. In the first training session,

the novel arm was blocked with a removable wooden door and the animal was given free access to the "start" and "other" arm for 15 mins of exploration.

In order to begin the trial session, the animal will be placed in the start arm, with its head facing away from the center (i.e. head was towards the wall of the start arm). At the end of the trial, the animal is removed from the maze and returned to its cage for 30 mins.

After the inter-trial wait, the animal will be returned to the maze with the wooden door removed and novel arm exposed. During the transfer the animal's head will face towards the center of the maze and will be placed in the "start" arm, and allowed to freely explore the maze for 5 mins. The trial test will be recorded using a video camera, and the video will be analyzed to record the number of time the animal entered each arm, along with the duration of time spent in them. The percentage choice of entry between novel and other arm was also calculated. After each animal and between the trials, the maze will be thoroughly wiped with 70% ethanol to prevent any odor cues.

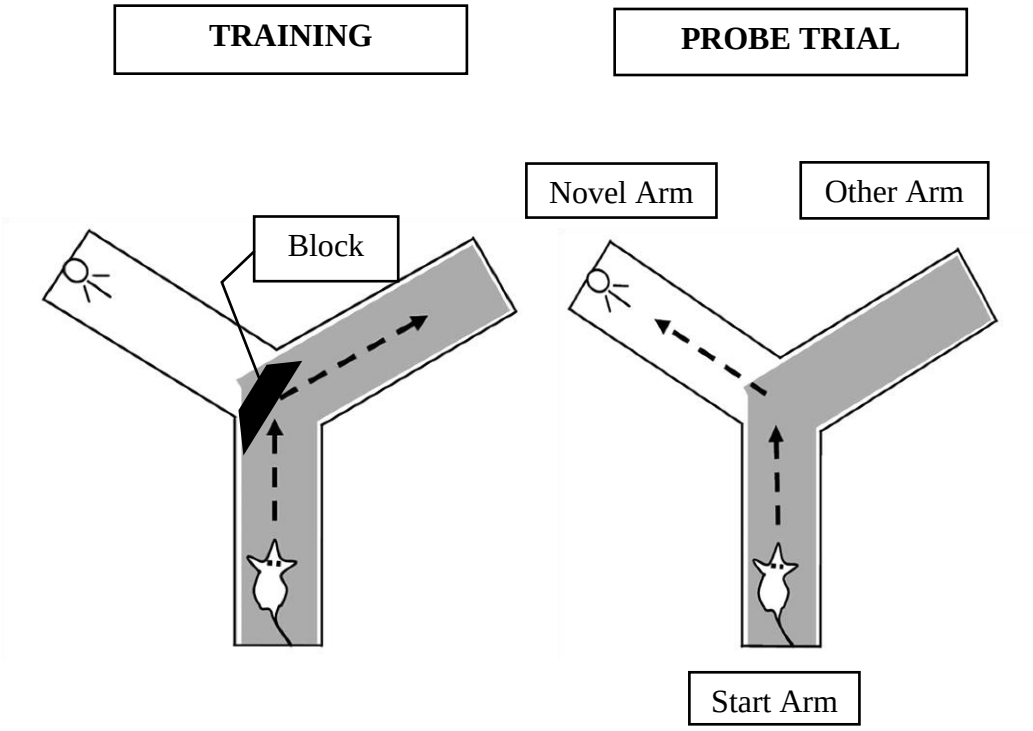

Figure 2.3: Diagrammatic representation of Y maze test.

2.6.1.2 Morris water maze test (MWM Test)

Purpose of MWM Test

Originally developed by Richard G. M. Morris (Morris, 1984), this test is of spatial learning and reference memory for rodents that depends on distal cues to circumnavigate from start locations of an open swimming arena and locate a submerged platform for escape. The protocol for this test was described previously by Bromley-Brits et al., 2011, with a few modifications. The test will be performed in a circular swimming tank with the water temperature maintained at 23±2°C.

Procedure

Training

The rats will be trained for 4 consecutive days to find the hidden platform in the tank. In order to train the rats, five trials will be conducted each day. During each of these trials, the rats were released in the tank from different directions (Table 4). An inter-trial interval of 10 mins will be given to each rat before each trial. The duration of each trial will be 90s, during which the rat will be allowed to freely explore the tank and find the platform. In case the rats are unable to find the platform after 90s, they will be manually placed on it for an additional 20s. If they find the platform within 90s, and sat on it for at least 5s, the time will be recorded and the trial is considered over.

Probe Trial

A single probe trial will be performed after 2 days, where the platform is removed and the rats are allowed to swim in the tank, trying to find the safety platform for 90s. Using a video camera, the trial will be recorded for video analysis, later. Using the

video, the rat's number of entries into the target quadrant is recorded, along with the time spent in the target quadrant and the number of platform crossings.

Table 2.2: Direction of release, for all trials, in Morris Water Maze Test.

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

2.6.1.3 Hole Board Test

The hole board test can be used to quantify working memory, general working memory, and reference memory under different conditions following habituation to the test arena. Li et al. (2009) used the standard hole-board apparatus to carry out a hole board test. He filled three of the nine holes with food. In this case, working memory holds the information about which holes the mice have already checked and which holes have food in one trial. If the mouse visits a hole without food, and then within the same trial visits the same hole again, it has committed a working memory error (Li, et al., 2009). Reference memory, on the other hand, is a form of long-term memory and helps us create mental representations and associations. Spatial reference memory is important in animals such as rodents due to foraging. Being able to use the environment to retrieve information and routes to find food allows them to survive (van der Staay, et al., 2012).

The holeboard apparatus is an open-field box, with a 16-hole floor insert. Same four holes are baited throughout the trials, which the rodents are expected to learn. After habituation to the apparatus, the rodents undergo four to six trials per day. The procedure for this test was adapted from (Kuc, et al., 2006), with slight modifications. The test was performed in an open field box with the hole board fixed inside, at room temperature.

Procedure:

Habituation

Mice feed was removed a day prior to the habituation and they were shifted in the testing room to acclimatize to the environment. The hole board was placed in the testing room and each of the 16 holes were baited with 100mg of food pellet.

Habituation was carried out in 2 trials. The first trial took place after an hour, of removing the mice feed. The mouse was placed on the board for 15 minutes, and allowed to freely explore each hole. After 15 minutes, it was removed from the box and placed back in its cage. The same procedure was repeated for the remaining mice. After an inter-trial interval of 3 hours, the same procedure was repeated as trial 2 for habituation.

Training period

The training period began the following day, (Day 1) and continued for 4 days. Each of the daily training sessions consisted of 4 trials, of 3 minutes each, with an inter-interval of 30 minutes. The start position of the mouse was randomly changed for each trial. Out of 16, the same 4 holes were baited with 300 mg pellets. The trial session ended when either, 3 minutes had passed, or the mouse had eaten all pellets from each of the baited hole. After the trial ended, the mouse was returned to its cage, the apparatus was cleaned thoroughly with 70% ethanol, to homogenize any olfactory cues left behind, and the eaten pellets had been replaced with newer ones.

By the end of the experiment, the Working-memory errors (entries to holes already visited during a trial), Reference-memory errors (entries to non-baited holes), Latency to visit the first hole, and activity per minute were calculated.

Table 2.3: Parameters for Hole Boarrd test with their purpose and description

MEMORY	PARAMETER	DESCRIPTION	PURPOSE
Long term memory (Reference memory)	Wrong choice	Any visit to non-baited hole; the nose below the rim	To check degree of memory impairment and/or effect of treatment on learning and memory
Short term memory (working memory)	Recurrent choice	Revisit to baited hole; nose below the rim	
Latency	Time taken to visit first hole	Time taken to visit the very first hole once the mouse enters the arena. The hole may be baited or unbaited	Delay in latency shows higher anxiety levels in subject.
Activity	Nose pokes/ Head dips	No. of head dips or nose pokes in a minute	To check exploration related behaviour
Omission Error	Omission of baited hole	No visit to baited hole	To check degree of memory impairment and/or effect of treatment on learning and memory

2.6.2 Serum profiling

2.6.2.1 Liver and Kidney Function Tests

Liver and kidney profiling was done as an initial screening tool for liver dysfunction. The results can give approximate estimation of certain genetic diseases, cardiovascular disease, and some other diseases. The profiling was done in ASAB diagnostic lab in order to assess the effect of metals and HFD on lipids and cholesterol level of animals.

2.7 Statistical Analysis

Statistical analysis was done by GraphPad Prism software (Version 5.01). The statistical tests applied to analyze the data were One-Way Anova, Two-Way Anova and the Bonferroni multiple comparison test. The significant P value was noted to be less than 0.05. The data was shown as mean ± standard error of mean (SEM).

Chapter 3

RESULTS

3.1 Behavioral Analysis

To check the effects of shogaol on learning and memory, in male Balb/c mice, various behavioral tests were conducted, which included Morris Water Maze, Y- Maze and Hole Board test.

3.1.1 Morris Water Maze Test

Morris water maze test, a long term memory test for 10 mice (per group) was employed, in order to check the influence of Shogaol on memory deficits caused by metals and HFD. The effects of the compound on spatial memory can be observed directly by comparing the average escape latencies between different groups. The outcome of both Shogaol concentrations (2mg/kg and 12 mg/kg) shows improvement of memory and learning, similar to control group. Mice exposed to high fat diet and metal water, with no treatment show an impaired performance, with the average escape latency not improving, significantly, over a period of 5 days training, as compared to the remaining three groups (Figure A). Learning deficits can be observed from day 2 where a significant difference can be observed between the learning of the metals+HFD group and metals+HFD+Shogaol (2mg) group.

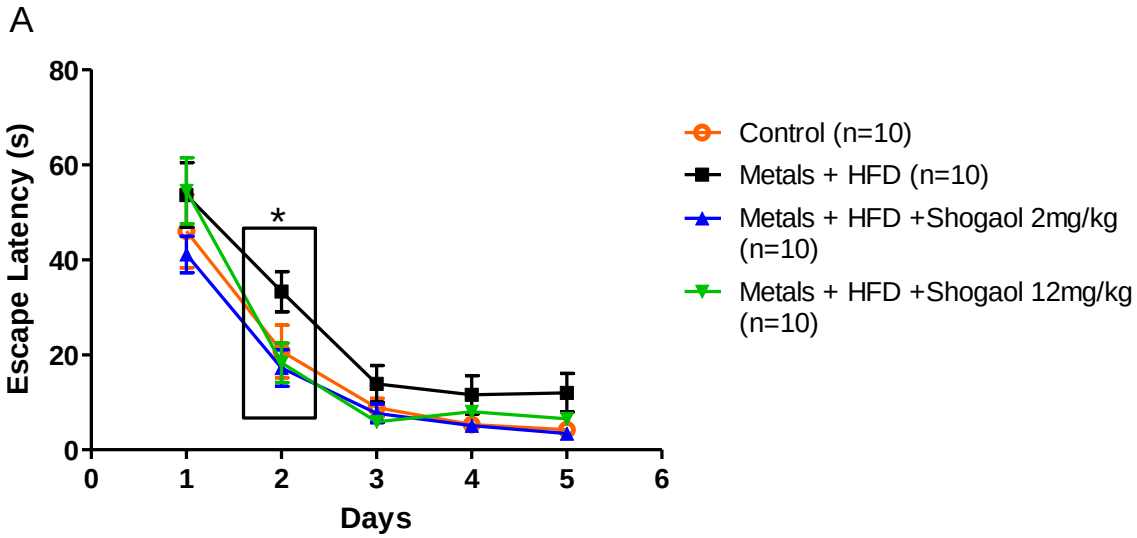

Figure 3.1: Effect of Shogaol on learning and memory using Morris Water Maze test; training. The graph demonstrates the escape latency (s) to assess the reference memory formation and learning among the control, metals+HFD, metals+HFD+Shogaol (2mg), and metals+HFD+Shogaol (12 mg). Error bars represent mean ± SEM.for two-way ANOVA, followed by Bonferroni's multiple comparison test. * $p < 0.05$, s= seconds. ***** metals+HFD vs. metals+HFD+Shogaol (2mg)

Reference memory was assessed by probe trial test (Figures B-D), performed after 5 days of training. In order to check the exploration activity, time spent by the mice in target quadrant was observed, in order to locate the (now removed) hidden platform (figure B). The control group spent the most time in the target quadrant (37.90 ± 3.57), and was compared to the remaining three groups. The metals+HFD group spent the least amount of time in the target quadrant, trying to locate the platform (28.60 ± 2.65), while metals+HFD+Shogaol (2mg) (35.20 ± 2.43) spent almost the same amount of time as the control group, and metals+HFD+Shogaol (12mg) (28.00 ± 2.02) treated group demonstrated results similar to the diseased group. The number of entries into the target quadrant were recorded (figure C) which showed no significant difference, however, the metals+HFD+Shogaol (2mg) (10.60 ± 0.476), showed the highest number of entries, followed by the Control (9.50 ± 0.69) group, whereas the metals+HFD group (8.40 ± 0.67) and metals+HFD+Shogaol (12mg) (8.50 ± 0.45) groups showed almost similar results, with the least number of entries in the target quadrants.

The number of platform crossings were recorded which showed significant improvement in the spatial memory for metal+HFD+Shogaol (2mg) (7.10 ± 0.55) group, with a p value of < 0.05; as compared to the metals+HFD group (4.40 ± 0.52) and metals+HFD+Shogaol 12mg (5.00 ± 0.63) group which showed the least number of platform crossings. The control group (6.70 ± 0.76) showed similar results to metals+HFD+Shogaol 2mg group.

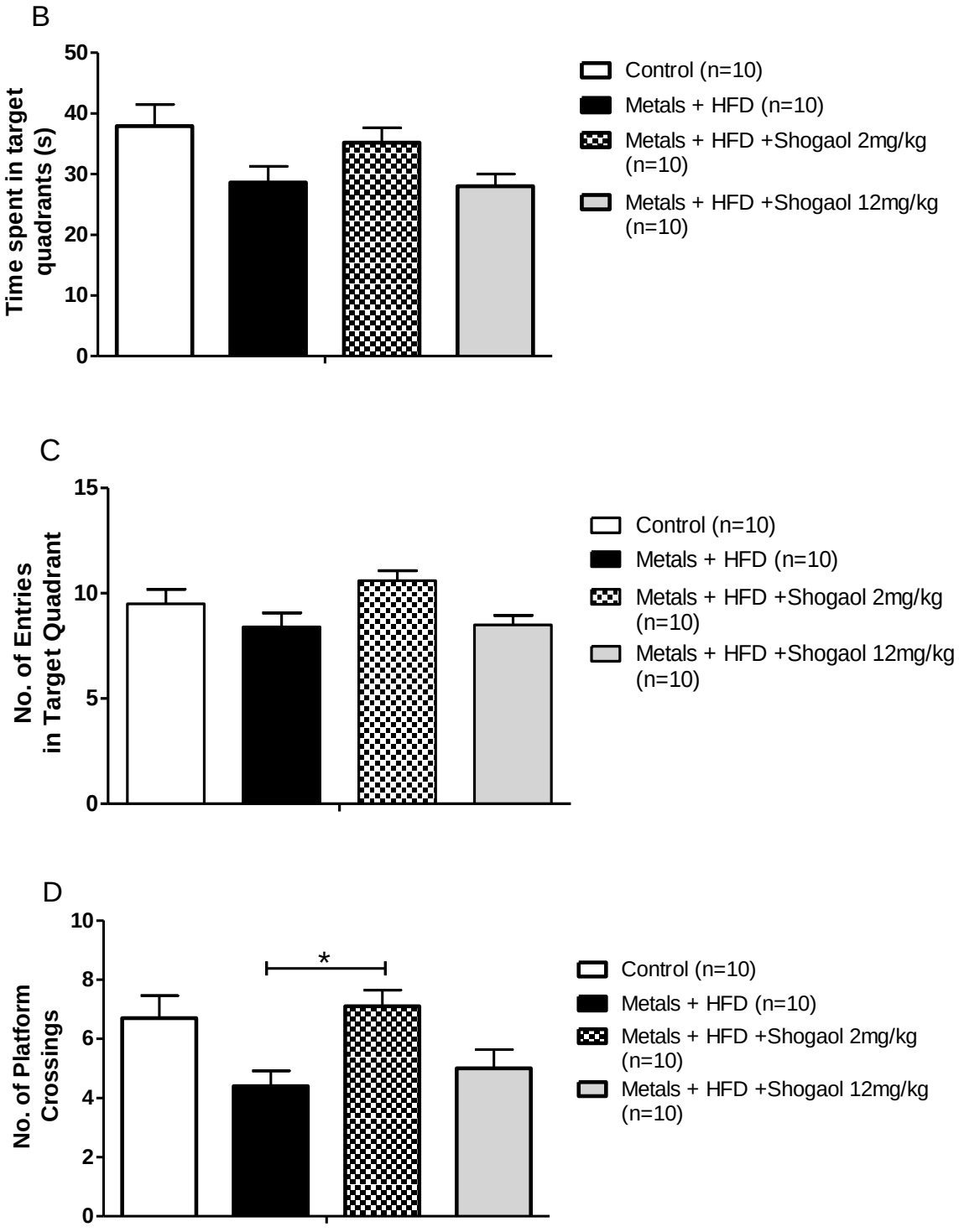

Figure 3.2: Morris Water Maze; Probe Trial. The bar charts depict (B) the time spent in the target quadrant (s), by all groups; (C) the number of entries in the target quadrant; (D) shows the number of platform crossings. The error bars are representatives of mean ± SEM for One-way ANOVA, followed by Bonferroni's multiple comparison test with * $p<0.05$ significant values. s = seconds.

3.1.2 Y Maze Test (Spontaneous Alternation Test)

Y maze test is based on the rodents' natural drive to explore novel environments and test the short-term spatial recognition memory. This simple behavioral task tests cognitive function based on the ability of the subject to remember spatial locations requiring hippocampal-dependent reference memory. The test was employed to assess the effect of Shogaol on exploratory behavior and recognition memory of the rodents. The test consists of two sessions; first one being the trial, while the second one is the probe trial.

All groups, i.e. Control (6.53 ± 0.84), metals+HFD (5.90 ± 0.85), metals+HFD+Shogaol (2mg) (9.27 ± 1.14), and metals+HFD+Shogaol (12mg) (6.90 ± 0.57) showed preference towards the novel arm, however mice treated with 2mg/kg Shogaol seem to have a significant improvement in exploratory activity and recognition memory (Figure A and B), as compared to the other three groups hence the increased number of entries in the novel arm. Both, metals+HFD+Shogaol (2mg) (96.97 ± 41.87) group and metals+HFD+Shogaol (12mg) (93.90 ± 40.79) group showed an increased preference towards spending more time in the novel arm, as compared to the Control group (92.17 ± 25.10), and metals+HFD (92.07 ± 29.99).

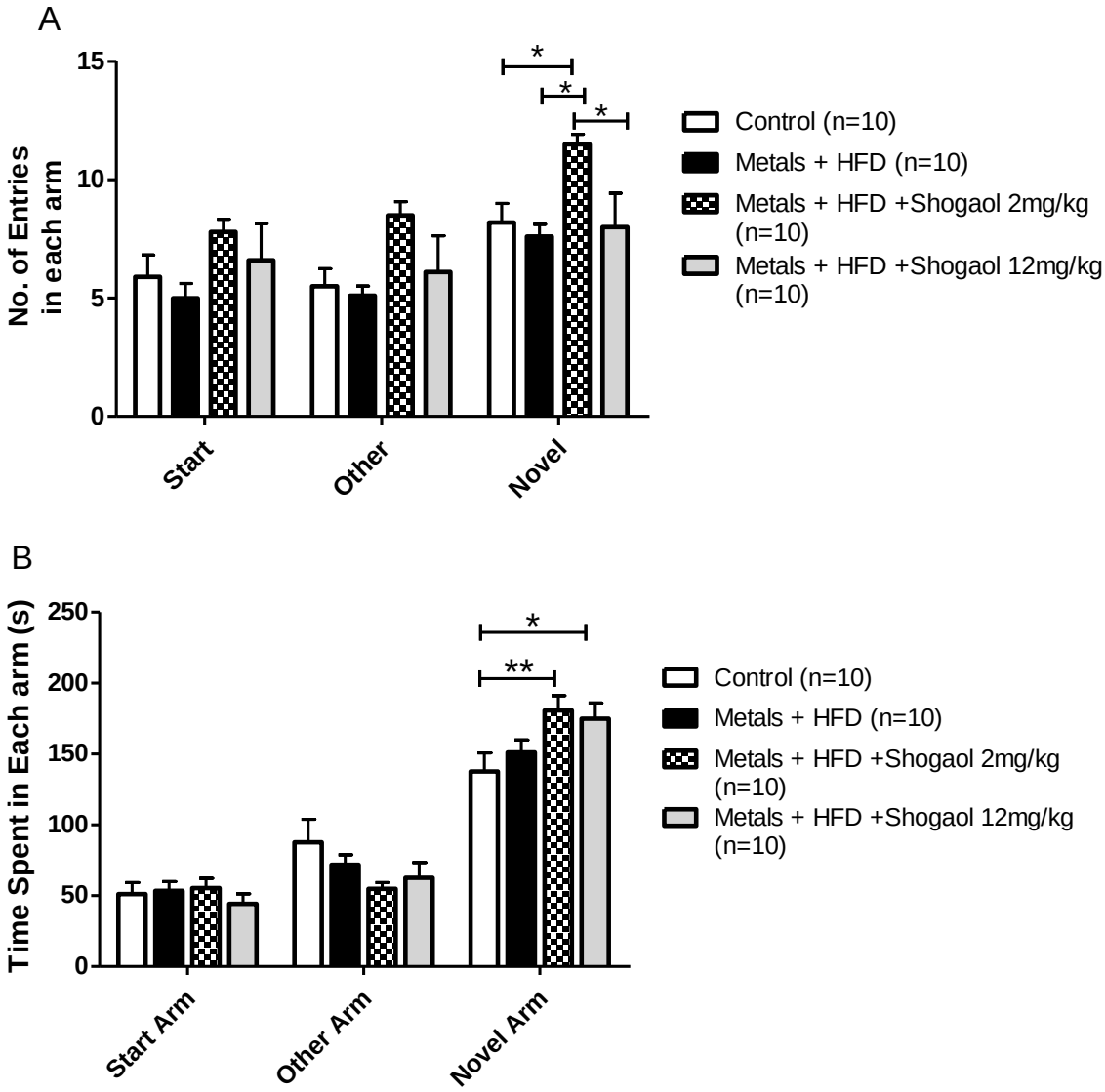

Figure 3.3: Performance of animals in Y-Maze test. The bar charts (A) Depict the number of entries in each arm, and **(B)** Depict the time spent in each arm (s), by Control, metals+HFD, metals+HFD+Shogaol (2mg), metals+HFD+Shogaol (12mg). Error bars are represented as mean± SEM, for two- way ANOVA, followed by Bonferroni's multiple comparison test. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ are the significance values. s= seconds.

To evaluate the degree of spatial memory impairment in all groups, three different parameters were assessed (Figure C-E). Spontaneous alternation performance test (Figure C) showed memory impairment in metals+HFD group (43.34 ± 2.33), as compared to the Control (56.99 ± 4.07), metals+HFD+Shogaol (2mg) (60.33 ± 2.67), and metals+HFD+Shogaol (12mg) (65.28 ± 4.33). Metals+HFD+Shogaol (2mg) group showed a significant improvement, with a p value of < 0.01, while the results of metals+HFD+Shogaol (12mg) group gave a p value of < 0.001.

Alternate arm repeats (AAR) (%) and same arm repeats (SAR) (%) were calculated to determine the degree of short term memory impairment in the mice. In figure D, metals+HFD group showed the highest number of AARs (46.48 ± 1.790), showing increased memory impairment, while the control group (33.69 ± 3.11) and metals+HFD+Shogaol (2mg) (33.65 ± 2.70) showed almost similar results when compared to metals+HFD group, with a p value of <0.01. However, the group treated with 12mg/kg of Shogaol (28.89 ± 1.956) showed the least number of AAR (%), depicting least amount of memory impairment, with a p value of < 0.001.

A similar trend is observed in figure E, where Control (0.0 ± 0.0) showed no same arm repeats (SAR), whereas, metals+HFD (3.43 ± 1.08) showed the highest number of SAR (%) suggesting increased memory impairment. Metals+HFD+Shogaol (2mg) (0.30 ± 0.30) give a p value of < 0.01, and metals+HFD+Shogaol (12mg) (0.0 ± 0.0) gives a p value of < 0.001, suggesting improved memory.

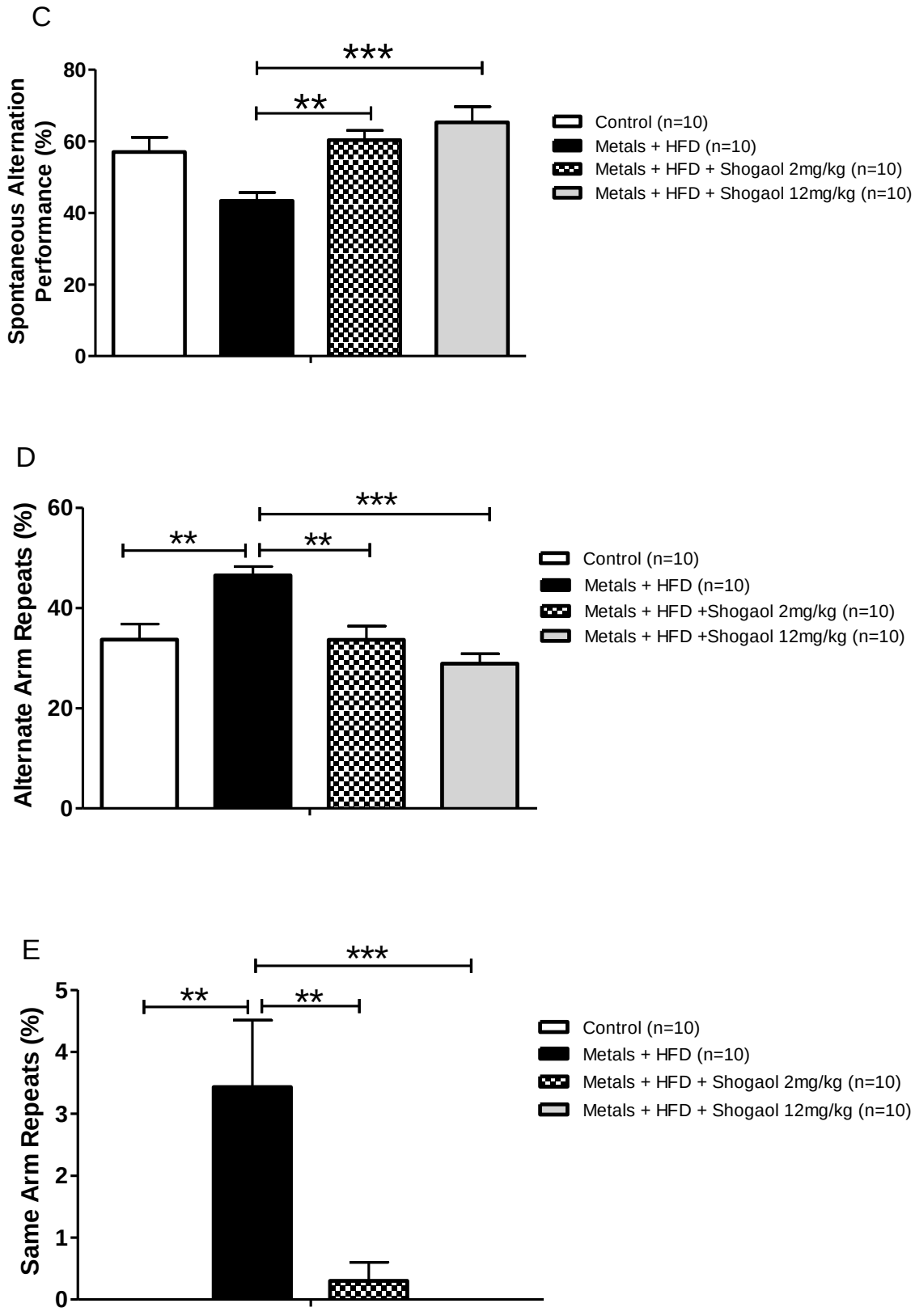

Figure 3.4: Effect of metals+HFD on memory impairment. (C) Spontaneous Alternation (%) in all groups, (D) shows the Alternate arm repeats (%) (E) **S**hows the same arm repeats (%), by the groups Control, metals+HFD, metals+HFD+Shogaol (2mg), metals+HFD+Shogaol (12mg). *= p< 0.05, **= p< 0.01, ***= p< 0.001 are the significance values. Error bars are represented as mean± SEM.

3.1.3 Hole Board Test

Hole board test was employed, in order to determine the effects of metals and HFD on long term and short term memory and locomotion activity. The Activity per minute of the mice was assessed to check the locomotion activity of the mice (Figure A). On Day 1, the Control group (3.56 ± 0.43) showed no significant difference from metals+HFD group (3.08 ± 0.29), however the control group showed slightly higher activity thus exhibiting better locomotion activity. The metals+HFD+Shogaol (2mg/kg) group (2.69 ± 0.38) and metals+HFD+Shogaol (12mg/kg) group (4.55 ± 0.41) showed significant difference, with a p value of < 0.01, with the metals+HFD+Shogaol (12mg/kg) group showing higher activity per minute thus better locomotion. The metals+HFD+Shogaol (12mg/kg) group exhibited a significantly higher activity, compared to the metals+HFD group, with a p value of < 0.05. On Day 4, a similar trend was observed, with the control group (1.96 ± 0.36) exhibiting better locomotion activity compared to the metals+HFD group (1.64 ± 0.17). Metals+HFD+Shogaol (2mg/kg) group (1.47 ± 0.29) showed the slowest locomotion activity amongst the three groups while metals+HFD+Shogaol (12mg/kg) group (2.35 ± 0.28) showed the highest locomotion activity.

Time taken by each mouse, to visit the first hole (whether baited or unbaited), was also recorded to determine the anxiety levels in the mice and also to check their locomotion activity (Figure B). Two-way ANOVA test was used to determine the difference between the activities on all days. The control group (15.71 ± 2.60) showed no significant difference from the metals+HFD group (27.07 ± 1.67), however showed lower anxiety levels compared to the diseased group. The metals+HFD+Shogaol (2mg/kg) group (23.68 ± 5.72) showed higher levels of anxiety on Day 1, however, the metals+HFD+Shogaol (12mg/kg) (12.31 ± 1.69) portrayed

least anxiety. A significant difference can be observed between the metals+HFD group and metals+HFD+Shogaol (12mg/kg), with a p value of < 0.05. On Day 4, all four groups followed the same trend. The control group (25.35 ± 7.21) showed decreased latency (s) compared to the metals+HFD group (34.81 ± 6.79), with a significant difference of < 0.05. The metals+HFD+Shogaol (2mg/kg) group (32.19 ± 5.21) showed decreased latency (s) compared to the metals+HFD group, while metals+HFD+Shogaol (12mg/kg) group (21.03 ± 6.57) showed the least latency compared to all three groups, and gave a p value of < 0.01, when compared to the metals+HFD group.

The reference memory errors were noted for all four days (Figure C). Two-way ANOVA test was employed for the test, to compare the learning throughout the trials (Figure C). At Day 1, while there was no significant difference between the control (5.81 ± 0.96) and metals+HFD group (8.48 ± 1.14), a difference can be seen, with the metals+HFD group showing a higher number of reference memory errors as compared to the control group. A significant difference can be observed between metals+HFD+Shogaol (2mg/kg) (5.10 ± 0.80) and metals+HFD+Shogaol (12mg/kg) group (9.77 ± 1.11), with a p value of < 0.05. On Day 4, the same trend was observed, however, a significant difference, was observed between the control group (2.70 ± 0.33) and metals+HFD group (4.42 ± 0.56), with a p value of < 0.05. A significant difference was also observed between metals+HFD group and metals+HFD+Shogaol (2mg/kg), with a p value of < 0.05, with the metals+HFD+Shogaol (2mg/kg) group showing better results with a lower number of reference memory errors. Another significant difference was also observed between metals+HFD+Shogaol (2mg/kg) group (2.29 ± 0.43) and metals+HFD+Shogaol (12mg/kg) group (5.00 ± 0.70), with a

p value of < 0.01, with the metals+HFD+Shogaol (2mg/kg) group depicting better performance with a decreasing number of reference memory errors.

The working memory errors were recorded for all four days (Figure D). Two-way ANOVA test was employed for all days, to compare the deficits in short term memory and learning at the end of the test. At Day 1, while no significant difference between the control (1.22 ± 0.29) and metals+HFD group (0.68 ± 0.17), a difference can be seen, with the control and metals+HFD+Shogaol (2mg/kg) group (0.78 ± 0.11) and metals+HFD+Shogaol (12mg/kg) group (1.28 ± 0.20). On Day 4, the Control group (0.79 ± 0.24) exhibited better working memory and showed reduced number of errors as compared to Day 1, while the metals+HFD group (1.00 ± 0.18), showed poor memory by having maximum number of working memory errors. A significant difference was also observed between metals+HFD group and metals+HFD+Shogaol (2mg/kg) group (0.28 ± 0.11), with a p value of < 0.05, with the metals+HFD+Shogaol (2mg/kg) group showing better results with a lowest number of working memory errors. The metals+HFD+Shogaol (12mg/kg) group (0.72 ± 0.12), showed results similar to the control group, with no significant difference.

Memory impairment was also assessed via omission errors (Figure E). Two-way ANOVA test was employed to compare the results for Day 1 and Day 4 as a comparison for learning. On Day 1, the control group (1.90 ± 0.24) showed significant results when compared to the metals+HFD group (3.23 ± 0.16), with a p value of < 0.001. While the metals+HFD+Shogaol (2mg/kg) group (2.60 ± 0.20) showed no significant results compared to the metals+HFD group, the metals+HFD+Shogaol (12mg/kg) group (1.85 ± 0.08) showed highly significant results compared to metals+HFD group, with a p value of < 0.001. On Day 4, although no significant difference can be observed between the values, the general

trend remains the same. The control group (2.62 ± 0.30) and metals+HFD group (2.64 ± 0.17) showed similar results, while the metals+HFD+Shogaol (2mg/kg) group (2.18 ± 0.15) and metals+HFD+Shogaol (12mg/kg) group (2.36 ± 0.19) showed the least number of omission errors, with the former exhibiting better results with the least number of omission errors, compared to the remaining three groups.

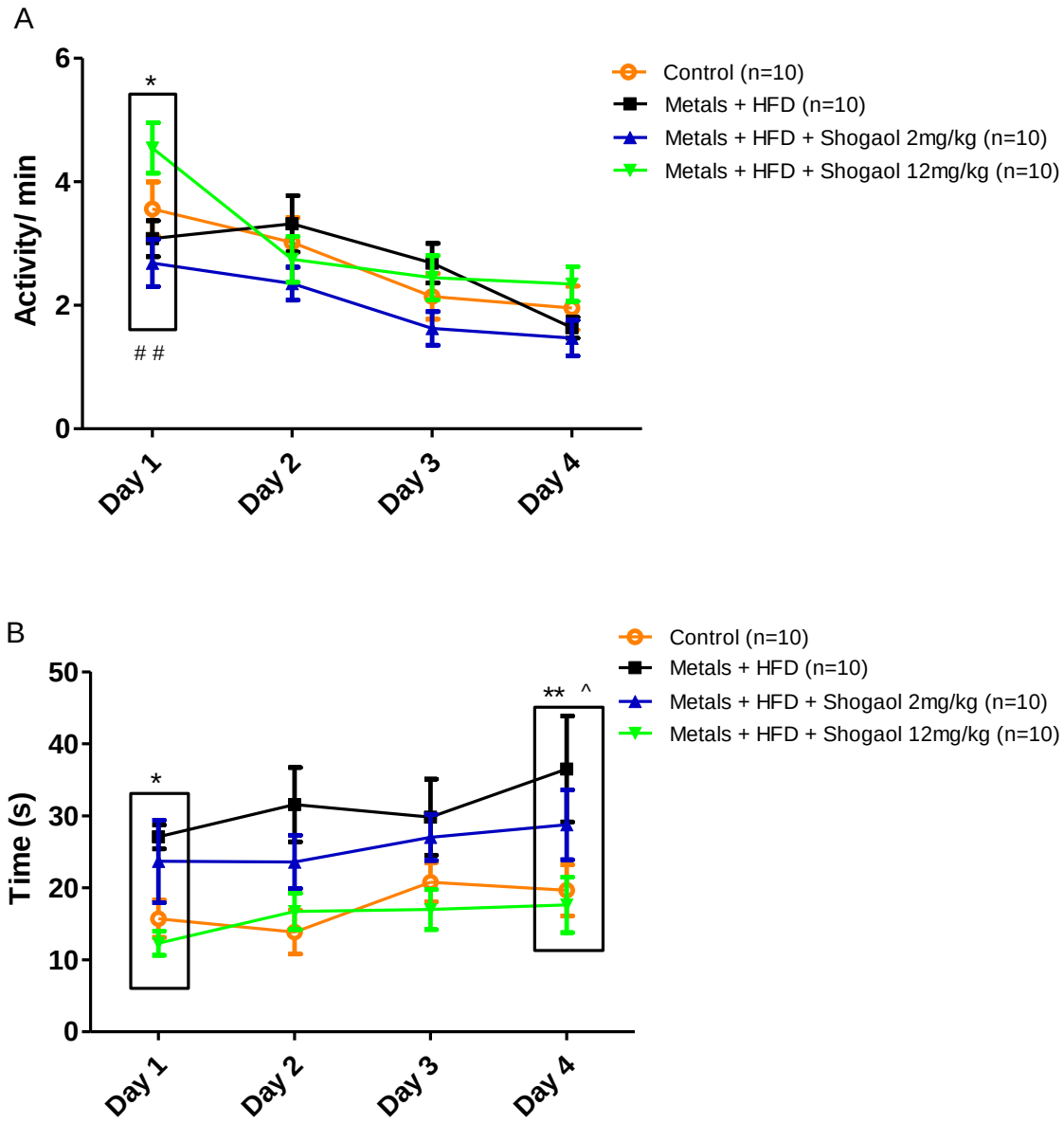

Figure 3.5: Effect of Shogaol on locomotion activity and anxiety levels. (A) Graph shows activity per minute, in all groups, (B) shows the latency (s) for visit to first hole,by the groups Control, metals+HFD, metals+HFD+Shogaol (2mg), metals+HFD+Shogaol (12mg). *= $p < 0.05$, is the significant value. Error bars are represented as mean± SEM. s= seconds, min= minutes.

^ Control vs. metals+HFD

* metals+HFD vs. metals+HFD+Shogaol (12mg)

metals+HFD+Shogaol (2mg) vs. metals+HFD+Shogaol (12mg)

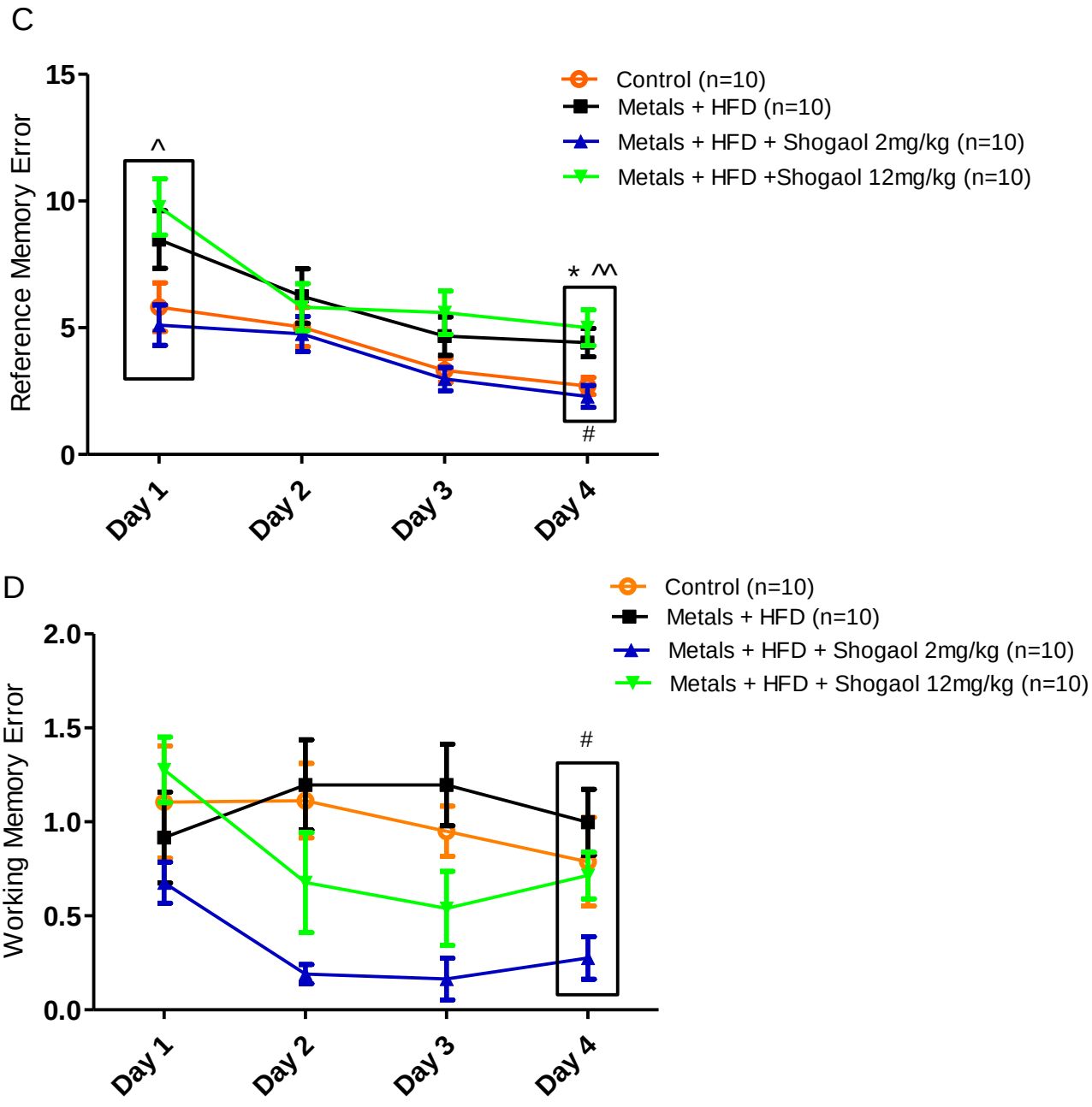

Figure 3.6: Effect of Shogaol on reference memory and working memory errors. (C) Graph shows reference memory errors, in all groups, **(D)**, shows working memory errors in all groups; control, metals+HFD, metals+HFD+Shogaol (2mg), metals+HFD+Shogaol (12mg). *= $p < 0.05$, is the significant value. Error bars are represented as mean± SEM. s= seconds, min= minutes.

* Control vs. metals+HFD+Shogaol (12mg)

metals+HFD vs. metals+ HFD+Shogaol (2mg)

^ metals+HFD+Shogaol (2mg) vs. metals+HFD+Shogaol (12mg)

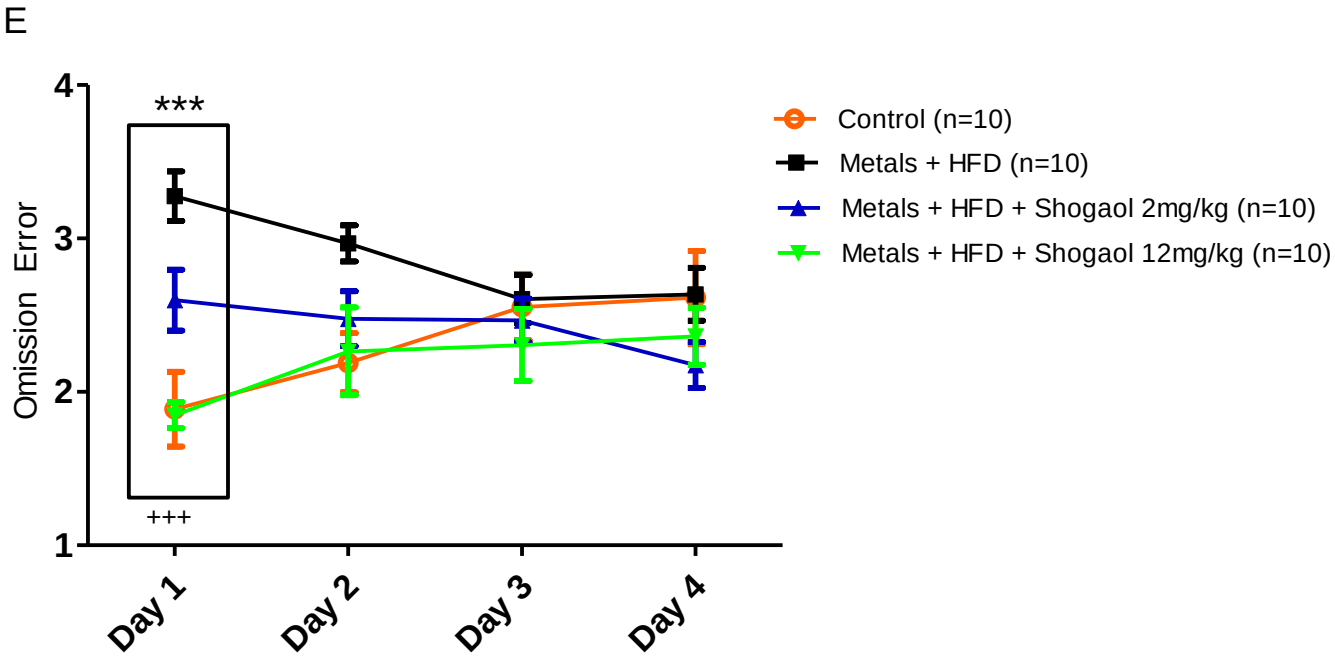

Figure 3.7: Effect of Shogaol on Omission errors. (E) Shows the omission errors by the groups control, metals+HFD, metals+HFD+Shogaol (2mg), metals+HFD+Shogaol (12mg). *= p< 0.05, **= p< 0.01, ***= p< 0.001 are the significance values. Error bars are represented as mean± SEM.

* Control vs. metals+HFD

+ metals+HFD vs. metals+HFD+Shogaol (12mg)

3.2. Biochemical analyzing

By the end of the study, the animals were sacrificed and their blood was collected for biochemical analysis, in order to check the effects of metals+HFD on various organs, mainly to check liver and kidney function impairment.

3.2.1 Liver Profiling

In order to test the degree of damage caused to the liver, levels of alanine aminotransferase and alkaline phosphatase enzymes, and total bilirubin were checked.

Alanine transaminase is a useful biomarker for liver injury or dysfunction. metals+HFD, metals+HFD+Shogaol (2mg/kg), showed the highest amount of ALT (92.80 ± 6.66), whereas, metals+HFD, metals+HFD+Shogaol (12mg/kg) groups (72.00 ± 8.14) and the control group (70.40 ± 2.38) showed almost the same levels. The metals+HFD group showed the least amount of ALT (59.20 ± 3.48), showing a significant difference with a p value of < 0.01. Alkaline Phosphatase enzyme levels were also measured as a measure of liver damage, due to metal exposure and HFD, the results of which showed a significant increase of the enzyme levels, in metals+HFD group (402.6 ± 85.08) with a p value of < 0.01, compared to the control group (85.80 ± 5.83). Both, metals+HFD+Shogaol group (2mg/kg) (201.4 ± 76.65) and metals+HFD+Shogaol (12mg/kg) group (185.8 ± 74.58), showed similar results.

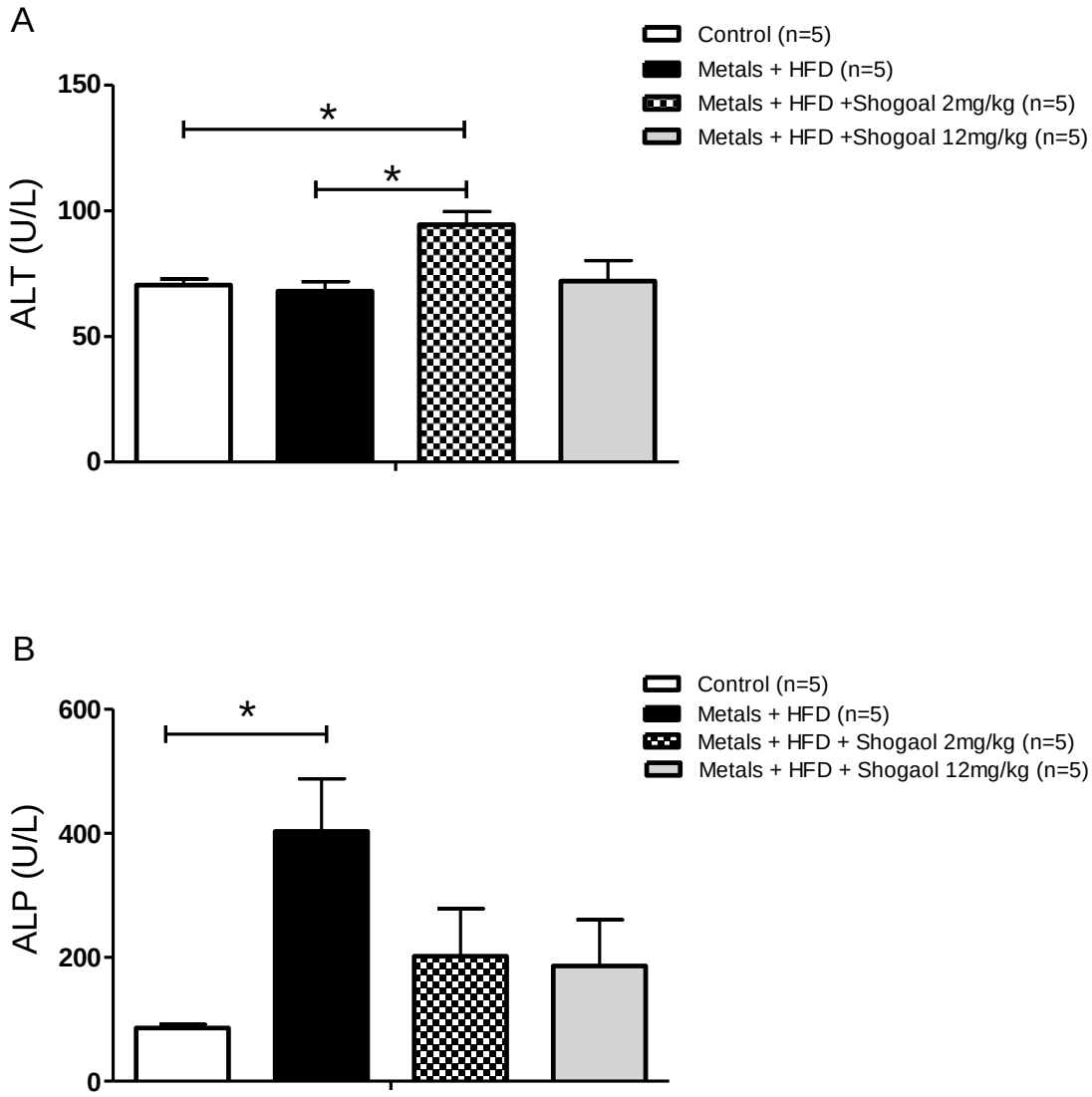

Figure 3.8: Effect of metals+HFD on liver function test. (A) The bar chart shows a ALT levels (U/L) compared between all groups. (B) The bar chart depicts the ALP levels (U/L) in all groups; control, metals+HFD, metals+HFD+Shogaol (2mg), metals+HFD+Shogaol (12mg). ******= $p < 0.01$, is the significant values. Error bars are represented as mean ± SEM.

3.2.2 Kidney Profiling

Serum Creatinine levels (mg/dL) were checked to determine kidney dysfunction. A general trend was observed were the control (0.22 ± 0.045) showed normal levels, while the metals+HFD group (0.37 ± 0.043) showed increased levels of creatinine levels in the blood serum, suggesting kidney dysfunction. Metals+HFD+Shogaol (2mg/kg) group (0.20 ± 0.051) showed results similar to the control, while metals+HFD+Shogaol (12mg/kg) group (0.31 ± 0.018) showed slightly reduced serum creatine levels suggesting an improvement in kidney function but not an effective dosage.

There was no significant difference between the levels of blood urea of all groups. The control group (55.60 ± 10.87) showed the highest concentration, while the metals+HFD group (38.20 ± 4.26) and metals+HFD+Shogaol (2mg/kg) group (40.80 ± 5.70) showed similar results that were the lowest amongst all groups. The metals+HFD+Shogaol (12mg/kg) group (50.40 ± 10.49) showed urea concentrations lesser than the control, however, more than the metals+HFD group and metals+HFD+Shogaol (2mg/kg) group.

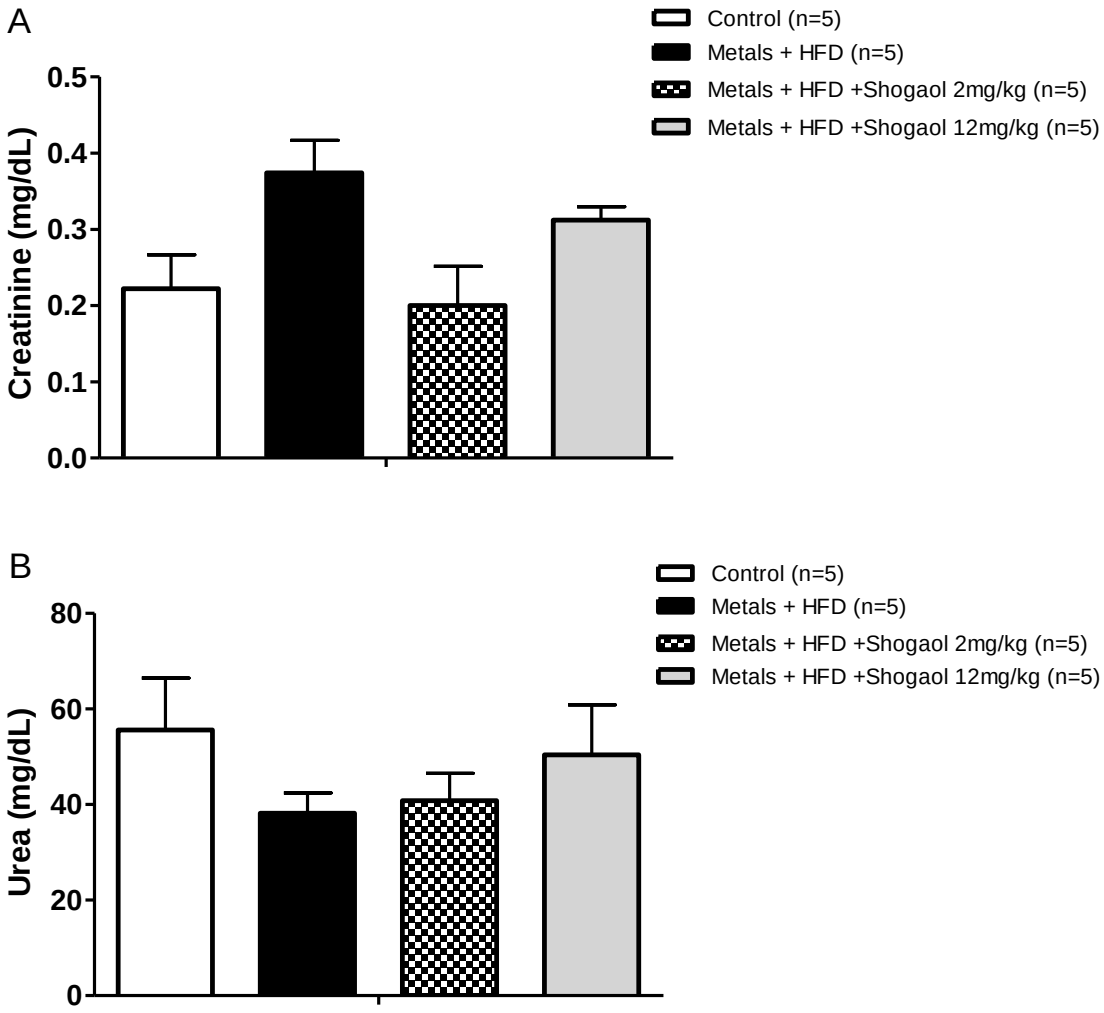

Figure 3.9: Effect of Shogaol on kidney function. (A) The bar chart shows a comparison of Creatinine levels of all groups. **(B)** The bar chart depicts the Urea levels (mg/dL) between all groups; control, metals+HFD, metals+HFD+Shogaol (2mg), metals+HFD+Shogaol (12mg). Error bars are represented as mean ± SEM.

Chapter 4

DISCUSSION

Being exposed through water, air and/or food, heavy metals remain a persistent threat to humans and the environment (Jan et al., 2015). Two main sources of for heavy metal pollution include anthropogenic and natural activities. An increase in multi-heavy metals pollution may be attributed towards biological buildup in the food chain (Wu et al., 2016).

Mejia et al., (1997) carried a research using a mixture of lead and arsenic, to determine its effects on the hippocampus. The study concluded that the hippocampus function is immensely affected by lead, whose mode of action is significantly enhanced by arsenic. Many studies have revealed that arsenic has extreme effects on neuronal synaptic functions in the hippocampus. Much like lead, arsenic inhibits the function of NMDA receptors, and up regulates the function of AchE.

Increased cases of obesity and overweight have been reported due to amplified intake of western diet (WD), causing major concern of institutions of public health. The most concerning factor about WD is not only it's easily assimilated carbohydrates and high content of saturated fats, but also the fact that such diets promote unorganized eating patterns, which consists of recurrent intake of either high energy food or plentiful meal before bed (Corwin and Hajnal, 2005). The term "comfort food" has been devised, as a concept closely linked to the effects of WD. The term refers to the consumption of HFD and food which causes appetite, to alleviate either stress, anxiety or both (Dallman et al., 2003).

Since such diets effect the permeability of the choroid plexus and blood brain barrier (BBB), WD is considered to have multifactorial effects on brain (Hsu and Kanoski,

2014). The effects also include inflammatory responses (Pistell et al., 2010), biochemical functions well-matched with neurodegenerative processes (Kanoski and Davidson, 2011), changes neurotransmission of both dopaminergic (Naneix et al., 2017) and glutamate receptors (Valladolid- Acebes et al., 2012) along with deficiency in neurocognition (Noble and Kanoski, 2016). Various clinical studies have indicated that obesity leads to deficits in overweight individuals of hippocampus-based declarative memory (Nilsson & Nilsson, 2009), also in the children and adults (Afzal and Gortmaker, 2015). Similarly, Yau et al., (2014), found that body mass index (BMI) and academic performance are inversely related.

Hippocampus along with its connections seem to be important in spatial learning and memory (Bird & Buggess, 2008). Studies conducted earlier have revealed that the any damage or removal of hippocampus affects the rate of learning. Both, learning and memory (spatial) are important attributes of survival instincts' in all the species. The ability to recognize and memorize allows flexible response to danger and the hallmarks of changing environment (Brandner, 2009). Information relevant to only specific trials for example retaining information about a recently visited place is called the memory of work or working memory, while the reference memory is known to be the ability to come up with solutions for a specific task and actions necessary to complete the task (Dudchenko, 2004).

Studies have linked use of HFD with an impaired functioning in hippocampal-dependent declarative memory, in both the humans and the rodents. For instance, when the rodents which were given normal diet were compared to the rodents that were given HFD projected an impaired various types of memory, also exhibited through poor performance results in Y-maze, (Almeida et al., 2017), and Morris water maze tests (Boitard et al., 2014).

The present study focused on the pharmacological evaluation of Shogaol in mice model against hippocampal memory impairment, caused by a mixture of metals and high fat diet.Four groups of animals were made, i.e. control which was given normal feed and water, while the remaining three groups were given HFD mixed in their feed and metals mixed in their drinking water. Two groups out of these three were receiving treatment by Shogaol at two different concentrations (i.e.2mg and 12mg) mixed in their feed. The behavioral changes were analyzed by performing different behavioral tests while some biochemical tests were also done to have a report of their effects on animals' metabolic profiles.

The most commonly and widely accepted test for spatial learning and memory is Morris Water Maze (MWM) test, the animal learns to escape from a round platform in this, originally intended to check the spatial reference memory, but modified procedure may allow the researcher to also assess the spatial working memory. The neural substrate of spatial orientation, particularly hippocampus and its connections are studied by applying all the spatial tasks. The hippocampal function is well indicated by MWM performance and it also elaborates cortex, striatum and cerebellum (Vorhees and Williams, 2006). In order to check the effects of HFD and metals on reference or long-term memory, MWM was conducted. Previously, a study conducted by Rodriguez et al. 2002, depicted little or no alterations in behavior, after a single metal exposure (arsenic) of 20mg/kg. Even though our study includes tertiary metal mixture, our results depicted similar behavior at 25 mg/kg exposure of each metal. The metals+HFD group portrayed longer escape latency in comparison with the control group & both Shogaol treated groups. However, over a period of five days training, the metals+HFD+Shogaol (2mg) group exhibited better performance with escape latencies closest to the control group, which may indicate Shogaol having a

positive effect on spatial reference memory but the effect cannot be deemed as significant. On the 6th day, a probe trial was conducted, where three parameters were recorded to assess the reference memory. Two parameters which included spent time in the target quadrant and no. of entries in that quadrant depicted no prominent results. Total no. of platform crossings showed a significant increase in metals+HFD+Shogaol (2mg) group, compared to metals+HFD group, suggesting improved spatial reference memory which may be attributed to Shogaol.

Rodent's natural exploratory activity is tested by Y-maze test. Both, spatial working memory & reference memory can be checked via this test. Rodents usually prefer to explore new arm of the maze instead of investigating previously visited arm. Consistent with our results for Morris water maze, showed less impairment while the spatial working memory showed significant impairment, however treatment with 2mg/kg of Shogaol showed significant improvement in both memories. To determine the impairment of reference memory, the spent time in novel arm and the no. of entries in that arm were noted. The no. of entries in novel arm was significantly more for the metals+HFD+Shogaol (2mg), in comparison with the remaining three groups. Metals+HFD group and metals+HFD+Shogaol (12mg), gave results similar to the control group, suggesting the reference memory was intact. Similar results were seen for spent time in every arm, were all four groups preferred to spend more time in novel arm in comparison with the remaining two arms. While the results again suggested that the long term memory was not affect by the Metals and HF diet, Shogaol still played effective role in increasing the reference memory, significantly. The metals+HFD+Shogaol (2mg) group showed an increase with a p value of < 0.01, while the metals+HFD+Shogaol (12mg) group gave a p value of <0.001, in comparison to the control group.

To assess short-term memory, via Y maze, three parameters were recorded, i.e. spontaneous alternation performance (SAP), number of the same arm repeats (SAR) & no. of the alternate arm repeats (AAR). Rodents generally tend to explore the arm visited least recently, and so, normally they are expected to alternatively visit the three arms. For this purpose, the rodents need to use their working memory, and so they need a maintenance and updated version of their record of the arm visited most recently (Wietrzych et al., 2005). Decreased no. of alternation indicate that the rodent cannot recall the most recently visited arm and thus, has an impaired spatial working memory, hence a decreased spontaneous alternation score. While the metals+HFD group showed the lowest SAP score indicating that they have impaired spatial working memory or short term memory, however, both the Shogaol treated groups showed highly significant improvement in the memory, with the metals+HFD+Shogaol (12mg) group showing better results as compared to metals+HFD+Shogaol (2mg) group. Working memory impairment was also observed via the SAR and AARs. The diseased group showed the highest percentage of AARs and SARs than the Control group. Both the Shogaol treated groups showed lower repeat percentages, though, metals+HFD+Shogaol (12mg) showed better results than metals+HFD+Shogaol (2mg), suggesting the higher dose of Shogaol is more effective in treating Metals and HFD induced neurotoxicity and improving spatial memory.

A spatial discrimination task for learning and memory was first reported by van der Staay et al., (1999) by employing the hole-board test. Spatial working memory and reference memory can be assessed with high reproducibility and low error by the use of hole-board apparatus. An animal's choice behaviour (visits and revisits to baited and unbaited locations) reflect the working and reference memory performance. Long and short-term memory systems were distinguished by Paul et al. (2009). Just like the

classification of Sharma et al. (2010), this classification system also includes a declarative memory component and and a long-term memory component. Meanwhile, working memory, a transitory limited capacity of holding current information, is thought of as a component of the system of short-term memory (Paul et al., 2009). In this perspective, using a hole-board relies on the hypothesis that animals which are exposed to a novel situation show a behavior resulting from the competition between exploratory and withdrawal tendency. Hence, a decreased behaviour of head-dips and oppositely, an increased behaviour of head-dips is manifested in increased and reduced anxiety, respectively.

Five parameters were recorded in the hole board apparatus. The activity per minute was a record for exploration related behaviour. Over a period of four days, the activity of all four groups seemed to decrease which suggests possible habituation of the mice. However, the overall locomotion of all groups seem to be unaffected by the administration of Metals and HFD. The time taken to visit any, first hole was recorded which was a parameter for checking the levels of anxiety in the test subjects. There was a significant difference between the metals+HFD and metals+HFD+Shoagaol (12mg) groups, suggesting Shogaol exhibiting a positive effect on anxiety levels in the mice. The mice in metals+HFD group showed increased latency on Day 1 and Day 4, suggesting increased anxiety during the trial sessions, while the Control group and metals+HFD+Shogaol (12mg) group, showed lowered anxiety levels, corresponding to lowered latency.

Furthermore, the test was used to analyse both, long-term and short-term memories, by assessing working and reference memory errors. The errors of reference memory seemed to decrease after a time period of four days, for all groups, suggesting learning and consolidation of memory, showing long term memory being intact for all groups.

However, Shogaol concentration for 2mg/kg seems to be the most effective against improving long term memory, significantly. In case of working memory, the errors increase for metals+HFD group, suggesting impaired spatial working memory. Both group treated with Shogaol show improved memory, by showing the least no. of errors of working memory. Visible decrease in the no. of errors can be observed in the graph. However, Shogaol, 2mg/kg enhances working memory significantly, compared to Shogaol 12mg/kg.

The effect of Metals and HFD were determined on liver and kidneys. At the end of behavioural study, the animals were sacrificed and their blood was taken for biochemical diagnosis. Liver and kidney function tests were done to determine the organ damage extent caused by the metals and HFD, and the role of Shogaol as a possible treatment option. There was no significant difference in the liver function tests among ALT levels and total bilirubin levels. The lowered ALT levels in metals+HFD group suggest either the Metal concentration was not enough to cause liver dysfunction or the given HFD was lower in concentration and did not produce any effect on the liver's function. The duration of study might also be short because, even though no significant difference was observed between the control group & metals+HFD group ALT levels. So it can be assumed the liver damage is at a very early stage, which is undetectable. Increased ALT and total bilirubin levels in both Shogaol treated groups, suggest possible toxicity caused by Shogaol, which eventually caused liver damage. Increased ALP levels in the blood also suggest liver damage; however it can also be indicative of bone and kidney damage. The increased levels of ALP, in metals+HFD group, suggest possible liver damage and suggestive of kidney and bone damage as well. The decreased levels of the enzyme in both Shogaol

treated groups indicate possible treatment however the treatment was not sufficient since the levels of enzyme were still more than that found in the Control group.

Advanced liver disease largely results in the pathological cause of reduced urea concentration (Kalhan, 2000). This indicates the central role of liver in urea cycle for urea production. Metal toxicity potentially targets mitochondria and their altered function can result in abnormalities in entire cells. Heavy metals' effects on mitochondria are illustrated; and in some instances the alterations in the functions of mitochondria have also been explained. Mitochondrial structure is altered in kidney by lead interactions (Nolan and Shaikh 1992). Some other studies have shown that PTP activated apoptosis is resulted from lead interactions with mitochondria (He et al. 2000). The current study has clearly indicated the association between kidney damage and renal function markers' alterations, which was seen after exposure to metals and HFD as an increase in the levels of creatinine. Albeit no significant difference was observed among the groups, results of both treatments showed effective inhibition of the increased renal function markers' levels and organ protection against Metals and HFD abnormalities.

CONCLUSION

Contaminated drinking water is a problem that every individual face in every part of the world in this world. Another problem is a HFD or commonly known as “Western Diet”. The present study showed that two of the most common problems of the world when combined, greatly impacts the ability of an individual to learn and memorize. Neuroprotective effects of Shogaol in mice model of Metals + HFD have been discussed in this study. Significant trends were observed in both Shogaol treated groups, in terms of memory improvement, however negative effects were observed in biochemical profile of liver fuctions suggesting possible toxicity at such doses. More research is required to estimate the effectiveness of Shogaol in HFD and induced neurotoxicity as well as the other biochemical abnormalities.

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