# Pharmacological Evaluation of Shogaol on Inflammatory Markers in Mice Model of High Fat Diet and Metals Induced Neuroinflammation


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In

Healthcare Biotechnology

By

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# THESIS ACCEPTANCE CERTIFICATE

It is certified that the final copy of MS Thesis written by **Ms. Rabia Basri** (Registration No. **00000206695**), of Atta-ur-Rahman School of Applied Biosciences has been vetted by the undersigned, found complete in all respects as per NUST statutes/ regulations, is free of plagiarism, errors and mistakes and is accepted as partial fulfillment for award of MS Degree. It is further certified that necessary amendments as pointed out by GEC members of the scholar have also been incorporated in the said thesis.

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Date: _____

Signature _____

Principal ASAB: **Dr. Hussnain A. Janjua**

Date: _____

# DECLARATION

I certify that this research work titled "**Pharmacological Evaluation of Shogaol on Inflammatory Markers in Mice Model of High Fat Diet and Metals Induced Neuroinflammation***"* is my own work. The work has not been presented elsewhere for assessment. The work here in was carried out while I was a post-graduate student at Atta-ur-Rahman School of Applied Biosciences, NUST under the supervision of **Dr. Touqeer Ahmed.** The material that has been used from other sources has been properly acknowledged / referred.

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**Rabia Basri**

*Dedicated to*

*My Parents & my Mamo Tehseen Ali for their*

*unconditional love and support*

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| | |
|---|---|
| Al | Aluminium |
| As | Arsenic |
| Pb | Lead |
| BBB | Blood brain barrier |
| GFAP | Glial fibrillary acidic protein |
| IL 1β | Interleukin 1 beta |
| TNF-α | Tumor necrosis factor alpha |
| ACh | Acetylcholine |
| AChE | Acetylcholinisterase |
| AD | Alzheimer's disease |
| PD | Parkinson's disease |
| ALS | Amyotrophic Lateral Sclerosis |
| ANOVA | Analysis of variance |
| ROS | Reactive Oxygen Species |
| W.H.O | World Health Organization |
| PPM | Parts per million |
| NO | Nitric oxide |
| AT | Annealing temperature |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| PCR | Polymerase chain reaction |
| SEM | Standard error mean |
| HFD | High fat diet |

| | |
|---|---|
| MRI | Magnetic resonance imaging |
| LPS | Lipopolysaccharide |
| EB | Evans blue |
| DMT-1 | Divalent metal transporter 1 |
| MAPK | Mitogen-activated protein kinase |
| PI3K | Phosphatidylinositol 3-Kinase |
| Tf | Transferrin |
| AlCl3 | Aluminium chloride |
| Cd | Cadmium |
| ER | Endoplasmic reticulum |
| CNS | Central nervous system |
| NMDA | N-methyl-D-aspartate |
| ERK | Extra signal regulated pathway |
| JNK | c-Jun N-terminal kinase pathway |
| MeHg | Methyl mercury |
| BMI | Body mass index |

## ABSTRACT

Neuroinflammation is complex pathological event in the central nervous system and spinal cord that involves the immune cells of the CNS including microglial and astrocytes cells. Humans are continuously exposed to metals from their surroundings and it is the prime cause of neuroinflammation. Neuroinflammation possess a key role in the progression of several neurodegenerative diseases. High fat diet intake is associated with obesity, cognitive dysfunction, ROS production which represent chronic inflammation. Upregulation of systemic inflammation due to high fat diet intake can recruit inflammatory mediators that can over-come the blood-brain barrier (BBB) and disrupt its functioning. Aluminium (Al), lead (Pb) and arsenic (As) are the most neurotoxic metals causing more harmful effects on the CNS when acting together than alone. The key objective of this study was to evaluate the anti-inflammatory role of Shogaol on metals+HFD associated BBB disruption and inflammatory cytokines. This study is designed to evaluate the effect of metals (Al, As, Pb) and HFD in mice model, 44 male BALB/c were divided into four group (n=11); Group1 (control), Group 2 (metals+ HFD), Group 3 (metals+HFD +Shogaol 2mg/kg) and Group 4 (metals+HFD+Shogaol 12mg/kg) Blood brain barrier assay was performed to check the blood brain barrier permeability induced by metals+HFD and the anti-inflammatory effect of Shogaol on the BBB. Quantitative expression analysis of the inflammatory markers (IL-1 beta, TNF-α and GFAP) was carried out in the cortex and hippocampus; it was found that metals+HFD significantly upregulated these inflammatory genes while Shogaol has the potential to normalize these inflammatory markers. However, further verification of the toxicity mechanisms underlying heavy metals and HFD is needed to further validate the result.

*Chapter 1*

# INTRODUCTION

Central nervous system is the main controlling system of the body. It has proper mechanisms for monitoring internal changes within a body and external environment (Marin and Kipnis, 2013). There are specific nerve filaments that extends towards the periphery and sense organs that screens the outer conditions of the body likewise for monitoring the chemical modifications within a body, specific receptors are located in the cerebrum. To maintain the balance of the body, nervous system works together with all other systems of the body and therefore CNS is called the main controller of homeostasis (Trenova et al., 2016).

There is a constant cross talk between nervous system and the immune system (figure 1.1), the peripheral immune cells that are present in the systemic circulation works continually for maintaining the normal homeostasis of the body (Jha et al., 2019). In case of any infection or injury to the body, peripheral immune cells reacts to external stimulus and fight with the infection at that site and as a result clear the infection. In spite of the fact that mutual effort between the two frameworks was for some time viewed as improbable due to their partition by the blood brain barrier (BBB), it is presently realized that such cooperation does happen, and in addition it is important for the normal physiological function of the body. Blood brain barrier is supposed to prevent the CNS from any injury or toxic materials (Koval et al., 1997). Immune cells resides in the CNS and both these systems communicate with each other, a proper signaling cascade occurs between nervous system and immune system. Thus, cells of the nervous system uses signaling by immune components with the help of different chemokines and cytokines (McAllister and van de

Water, 2009). Cytokines are basically the immune cells which initiate the immune responses that are required for normal function of the nervous system. Tumor necrosis factor (TNF-α) and interleukin-1 beta (IL-1β) are the cytokine known for its role in inflammation. These cytokines have a key role in the synaptic activity of the neurons and for the proper functioning of the nerve cells. T cells and residing immune cells are known for its role in learning and memory, also essential for higher cognitive functions (Marin and Kipnis, 2013).

Immune system and nervous system regulate each other tightly (Steinman, 2004).The nerve fibers or nerve filaments monitor the immune system and also check the level of cytokines in normal physiological condition on the other hand immune components (chemokines, cytokines and MHC) helps in neuronal separation and the development and adjustment of neuronal associations (Kasten-Jolly et al., 2012). Inflammation is a key feature in many neurodegenerative diseases Alzheimer's disease, Parkinson's disease, multiple sclerosis and other diseases like asthma, autoimmune diseases, cognitive impairment (Gonzalez-Scarano and Baltuch, 1999).

### 1.1 Neuroinflammation

Neuroinflammation is defined as the inflammatory reaction generated in the nervous system. It is basically an inflammatory response produced by innate immune system in the central nervous system and peripheral nervous system. The key mediators of neuroinflammation are astrocytes and microglial cells (Shaftel et al., 2008). This inflammatory response is initiated when the astrocytes and glial cells are activated. There are four phases of neuroinflammation. First astrocytic and microglial cells activation occur

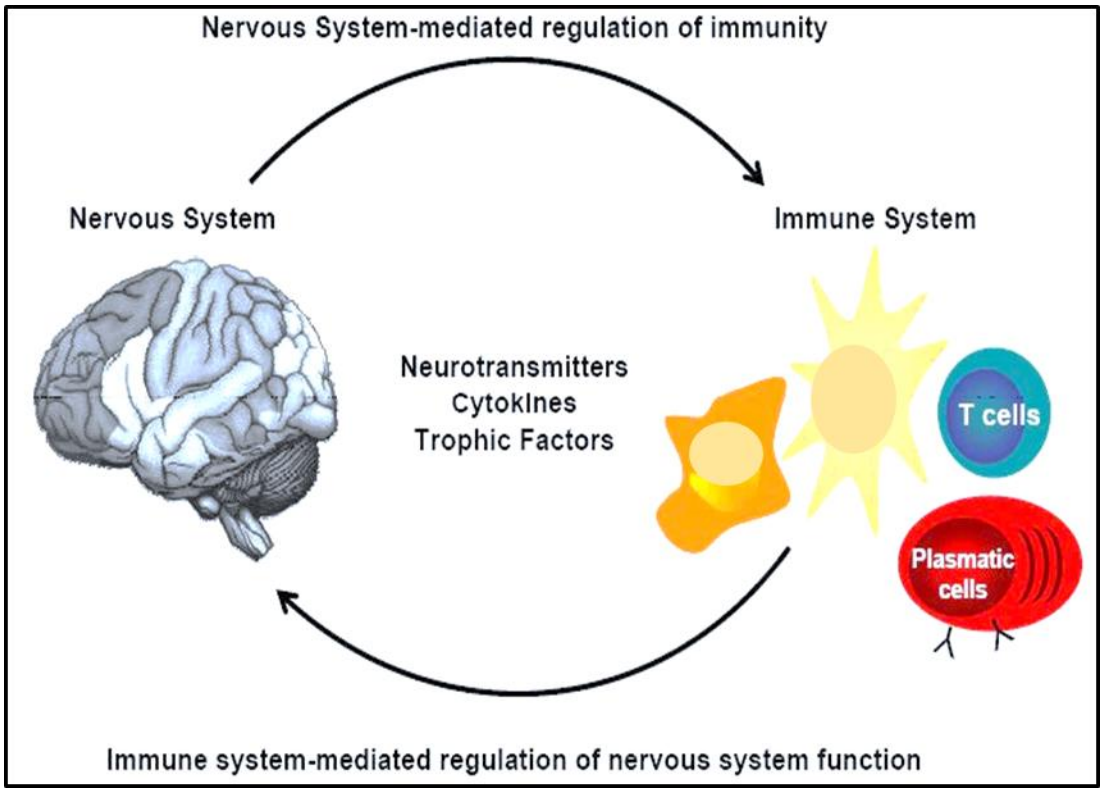



**Figure 1.1 Interaction between nervous system and immune system:** T cells, B cells, dendritic cells, plasma cells, various cytokines and different neurotransmitters participate in neuroimmune connection. Adapted from (Steinman, 2004).

as a result cytokines and chemokines (TNF α, IL 1β, IL 6) expression level increases which facilitates the inflammatory process. GFAP (Glial Fibrillary Acidic Protein) which is an astrocytic marker is also up regulated .When all this inflammatory response is initiated this leads towards oxidative stress in which reactive oxygen species and reactive nitrogen species are produced (Nakagawa and Chiba, 2016). It has proved that cytokines can pass the blood brain barrier. This transport of cytokines occurs by active means of transport or through the endothelium as the endothelia is compromised in diseased condition. CNS has been shown to be affected by both the inflammatory mediators present in the nervous system and the inflammatory cytokines from systemic circulation (Pollmächer et al., 2002). All these factors combine together which effect the structure and function of the BBB, its permeability increases, and inflammatory cytokines cross this barrier as a result neuronal injury occurs. There are various grades of neuroinflammation which relies on the time span, nature and condition of initial stimulus (Lyman et al., 2014).

There are two types of inflammation, acute phase inflammation and chronic inflammation. Acute phase inflammation is facilitated by production of initial response cytokines which includes TNF, IL-6 and IL-1β, inflammatory proteins, upregulation of adhesion proteins, increased production of chemotactic compounds (Ghirnikar et al., 1998, Da Cunha et al., 2010). In case of chronic inflammation there is an increase entry of immune cells from systemic circulation to brain which leads to swelling of tissues and might induce cell damage. Neurodegenerative disorders occurs when there is inflammation in the brain or spinal cord (DiSabato et al., 2016).

Microglial cells which accounts 10-20% of glial cells are known as local immune cells of the nervous system and play important part in the pathogenesis of various diseases like

neurodegenerative disorders which are related to inflammation (Elmore et al., 2014). In normal physiological condition, microglial cells are in relaxing state and have long processes by which these cells carefully observe the overall physiology of the nerve cells (Ginhoux et al., 2010). In the course of any injury to the CNS or infection, these glial cells change their morphology from round cell body to star shaped body (Figure 1.1) and thus are in activated state (Nimmerjahn et al., 2005). Activated microglial cells increase their number, phagocytose and initiates inflammatory response in which various mediators, toxic elements are produced. These toxic elements accumulates in the neuron which affects the structure and function of neurons as a result cell death occurs (Suffredini et al., 1999).

**1.2 Risk Factors of Neuroinflammation**

There are various risk factors that is associated with neuroinflammation. Microglial cells are activated due to aging, oxidative stress, tissue damage, infectious agents, pollution some neurotoxic compounds, environmental factors, exposure to fatty diet and mutations (Bradl and Hohlfeld, 2003). When microglia get activated, there is an increased secretion of inflammatory mediators which could be the potential risk factor for many diseases like neurodegenerative diseases via activation of many complex pathways of inflammation mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-Kinase PI3K/AKT pathways. These activated microglia are the source of ROS production, which activates nuclear factor kappa-light-chain-enhancer of activated B (NF-$\kappa$B) pathway and promote neuroinflammation, leads to neuronal death (Bradl and Hohlfeld, 2003).

Increased secretion of cytokines results in chronic inflammation which are the hallmark for many neurological diseases such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), multiple sclerosis (MS), amyotrophic lateral sclerosis

(ALS), tauopathies and age-related macular degeneration (ARMD) (Swaroop et al., 2016). Keeping in view all these factors of neuroinflammation, there is need of microglial inhibition to over- come neurotoxicity. Different targeted therapies are need to be discovered for neuroinflammation. Extraction of natural compounds and constituents of the natural compounds to be used as a new synthetic drug for suppression of inflammation (Chen et al., 2007).

### 1.3 Heavy Metals Toxicity

Heavy metals have a natural origin, but the anthropogenic activities based on technology and research have led to prominent changes in natural cycles. Hence resulting in accumulation in plants which intern have severe impacts on the body (Singh, 2007). The concentration of any heavy metals plays an important role on how it will affect the human health (Lane and Morel, 2000). For examples metals like copper (Cu), zinc (Zn) and cobalt (Co) are required in optimum concentration by the body but however if taken in higher concentration they may result in toxification (Chronopoulos et al., 1997)

Heavy metals affects human body by altering the metabolic functions, or by accumulation in vital organs including the kidney, liver, heart and bring changes in the normal physiological functions. Both plants and animals require heavy metals in trace amounts, the higher the concentration results accumulation in brain. They are responsible for causing different harmful intracellular activities like oxidative stress, dysfunction of mitochondria, DNA disintegration, protein misfolding, ER stress and abnormal activation of apoptosis (Strong et al., 1996, Wright and Baccarelli, 2007, Angeli et al., 2014).

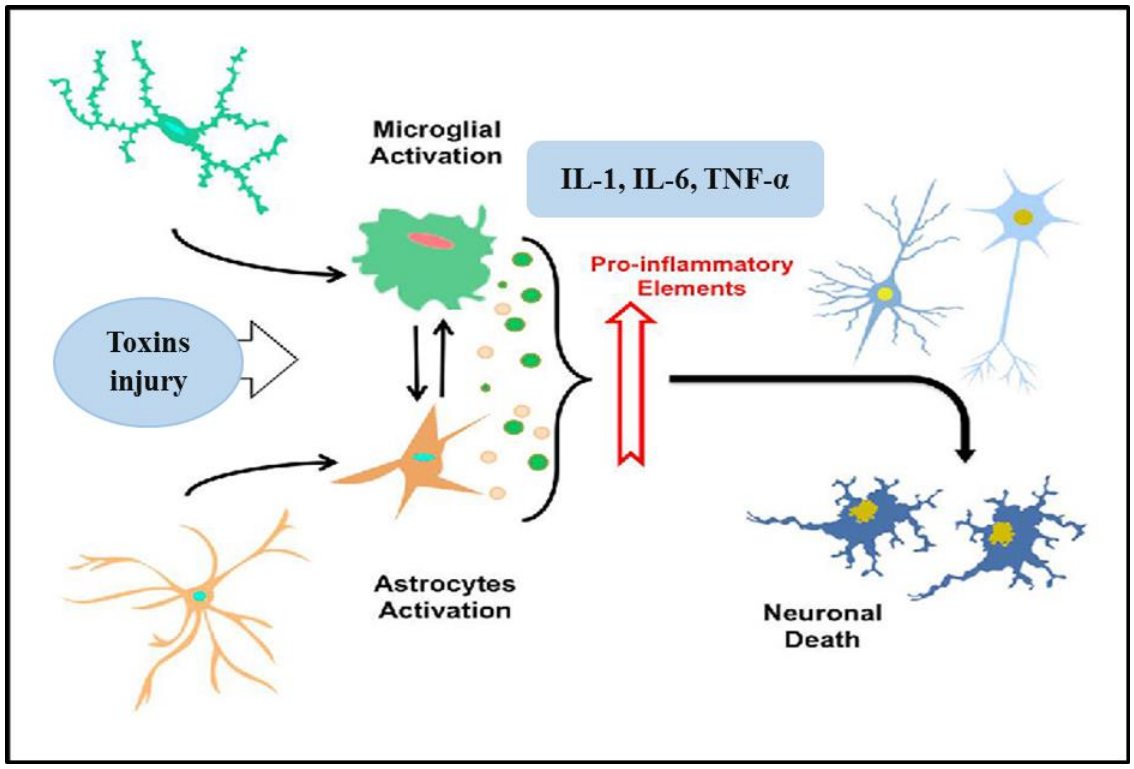



**Figure 1.2** Inflammatory response initiated by microglial cells results in neuronal cell death. Adapted from (Morales et al., 2014)

Such activities may be involved in bringing changes in the neurotransmission; hence resulting in various neurodegenerative diseases. It often leads to cognitive issues such as movement dysfunction, learning and memory impairment. Different neurological diseases such as Alzheimer's disease, Gulf War syndrome, Huntington's disease, Parkinson's disease play an important role in dating neurotoxicity (Desai and Kaler, 2008, Shaw and Tomljenovic, 2013).

Heavy metals can impacts the biological system in various ways. One of them is the blockage of the necessary activities taking place within the body. Although the environment can never be free from the presence of heavy metals completely. The uptake of heavy metal occurs via contaminated food, water and air. In case of Pakistan cadmium (Cd), arsenic (As) lead (Pb) and mercury (Hg) are present along with the ground and in water. Bioaccumulation is main reason behind toxicity among humans (Beyersmann and Hartwig, 2008). For technological advancement heavy metals are being explored in order to meet the needs of people. But it seems otherwise as heavy metals have become a new source of pollutants in the environment causing soil pollution, air pollution (Afridi et al., 2008). The human body is incapable to break down such heavy metals but does have the capability of efficient absorption, transportation and uptake procedure (Clemens, 2006). The heavy metals enter the body due to these capabilities. These metals while present in human body can lead to the systemic inflammation in the body in which different cytokines and mediators are produced, which disturbed the overall working of the body and affects the central nervous system (Jomova and Valko, 2011).

### 1.3.1. Mechanism of Metals Entry into the Brain

Metals that are mainly responsible or causing neurodegeneration are the metals that are required by the human body in small amount. These metals include copper, iron, manganese, and zinc. Concentration of heavy metals intake plays an important role as if these metals enter the body more than their required amount these can result in neurological disorders (Yokel, 2006). The heavy metals enter the body by overcoming the BBB and choroid plexus. The BBB divides the body form the brain from the rest of the body. On overcoming this barrier, the heavy metals move into the cerebrospinal fluid moving further various parts of the brain (Bhowmik et al., 2015). Although the BBB hinders the diffusion of various substances that are non-lipophilic but these free metallic ions and multiplexes combine with the amino acids resulting in hydrophilic nature for example transferrin which cannot be distribute by the BBB at enough rate (Wright and Baccarelli, 2007). The toxic metals possess the affinity for divalent metal ion transporter-I (DMT-I) and transferrin resulting in the important interactions in between the BBB and the brain tissues (Piloni et al., 2013). In case of brain development, the transportation of these metals occurs via systematic circulation by DMT-1 and transferrin (Karri et al., 2016). Metals enter into the circulation through absorption taking place at gastrointestinal track, lungs and skin. Other means of heavy metals being exposed to the body include sensory nerves present at nasal cavity and then these metals gain access to the BBB (Uthus and Seaborn, 1996).

### 1.3.2. Aluminium Toxicity

Aluminium (Al) is found is to be the third most rich element present on the earth's surface (Gupta et al., 2013). Al exits everywhere in the natural environment including air, water & soil. Different activities like mining processes and smelting out of Al from mines rises its

level in the environment (Fox et al., 2009). Main sources of Al exposure includes various products like paints, packaged food, perfumes, cosmetics products, hair dyes, medicines, drinking water & different types of vaccines (Rifat et al., 1990). Hence serving to be a major threat for living things i-e humans, plants and animals. Al is a strong neurotoxin causing cognitive deficits (Jaishankar et al., 2014b).

Long-lasting exposure of Al leads to neurologic deficiencies, representing degeneration of neurons, physiological alterations in the hippocampus, cortex and also alterations occurs in biochemical composition of nervous system. Diseases like Alzheimer's disease (AD) and Parkinsonism, dementia are still in debate (Järup, 2003). AlCl3 is responsible for impairment in learning, memory & cognitive function and locomotive deficits (Amjad and Umesalma, 2015). Al leads to increased expression of various pathogenic genes like IL1, IL-6, TNF-α, APP. Main region for Al accumulation is hippocampus and thereby affect the CNS (Roskams and Connor, 1990, Lukiw et al., 2005). Al has severe impacts on the immune system as well as CNS including deficiency of neurotransmission, loss of synapse, BBB dysfunction, activation of glial cells, neuroinflammation, irregular activation of transcriptional genes, neuronal damage and amyloid β formation occurs (Tomljenovic, 2011). Its role as the cause of dialysis dementia is well established (de Francisco and Luis, 2008). Disease known as dialysis dementia also occurs due to Al exposure. The symptoms of this disease are language difficulties, electroencephalogram variations, memory loss, muscles weakness and epilepsy occurs due to renal dysfunction (Banks and Kastin, 1989). Whereas greater serum concentrations of Al in elders results in impaired coordination and memory. Hence showing that neurotoxicity may occur despite normal renal function (Bowdler et al., 1979, Roskams and Connor, 1990).

There are different means of exposure of Al. In case of ions, chemical compounds, fine particulate matters of Al, exposure can occur via ingestion, inhalation and injection. After exposure it can reach different parts of the body through the blood stream damaging the BBB, immune system, central nervous system (olfactory nerve) causing immunotoxicity and neurotoxicity (Jaishankar et al., 2014a). This can impact the behavior of different animal model including rats and mice model. Al exposure activates complex signaling pathways that induces cell damage by the generation of ROS, abnormal cell division and tissue damage which leads to stimulate cytokine that interferes with DNA replication and alter various processes of the body (Niu et al., 2018).

### 1.3.3. Arsenic Toxicity

Arsenic (As) occurs naturally making combination with other metals. It is the 20$^{th}$ most abundant element located at the earth's crust. It is present in the environment due to release from the minerals via anthropogenic activities, weathering by wind and water solutions (Fulladosa et al., 2007). Exposure of human beings occurs with As is due to intake of contaminated water. Food poisoning occurs due to intake of food having pesticides and food grown with high concentration of As (Nriagu and Azcue, 1990, Hughes et al., 2011). As toxicity has been described all over the world. The permissible limit of arsenic for drinking water in countries such as US is 10 microgram per liter according to WHO. With reference to WHO, millions of people are exposed to As due to drinking water having large amount of inorganic arsenic (Edition, 2011, Jing et al., 2012). Greater concentration of As can lead to the suppression of N-methyl-D-aspartate (NMDA) receptors located in the hippocampus hence impacting the synaptic plasticity, learning and memory. This can intern cause delay in growth defects in neuronal tube. Whereas lower concentration of arsenic

than required can result in cognitive dysfunction (Carlin et al., 2015). As can pass the barrier present between the blood and brain and accumulate in the brain tissues which results in oxidative stress, upregulation of pathogenic genes, disturbed the scavenging activity of the cell, dysfunction of mitochondrial activities and neuronal death (Ramsey et al., 2013). The carcinogenic capability of As is well reported and studied using animal model. As when exposed to humans, complication occurs like low IQ level, attention deficits and have a well-established effect on cognition and memory (Luo et al., 2009).

### 1.3.4. Lead Toxicity

Lead (Pb) is also found in the earth's crust hence occurring naturally and is found to be very toxic. It is also present in environment due to anthropogenic activities such as mining, burning of fossil fuels and industrial processes (Roney et al., 2006). The main sources of Pb exposure are the different industrial activities, smoking fuel, contaminated drinking water, paints made of Pb, gaseous elements, cosmetics, toys, dust, emission from different industries, soil erosion etc (Thürmer et al., 2002). It is observed that Pb poisoning occurring due to drinking water. Those pipes carrying water is the main reason of water contamination (Brochin et al., 2014). Pb on exposure can intrude into the central nervous system as well as gastrointestinal tract and cause several diseases which affect children and adults (Markowitz, 2000). Pb exposure caused the astrocytosis and microgliosis which represent state of inflammation. Lead can intrude into the BBB interferes with calcium signaling and impairs excitation of neurons (Landrigan, 2000). It effects cholinergic system, deceased the activity of acetyl cholinesterase (ACh E) which is important enzyme in cholinergic system (Martin and Griswold, 2009). Pb can impact the human health in number of ways, but it depends upon age and the level of exposure. The main impacts of

high content lead uptake result in mental weakness, birth deficits, Attention Deficit Hyperactivity Disorder (ADHD), different allergies, impaired thinking ability, learning disorders, weight loss, movement disorders, muscles weakness, speech difficulties and kidney damage (Bradbury and Deane, 1993).

### 1.3.5. Combined Exposure of Metals

The elements occurring naturally in our environment, however imbalance exists due to anthropogenic activities taking place. It was observed that impacts of metals to affect individually is usually not common, but they may have greater impacts on co-exposure because of their synergistic or antagonistic activity producing physiological effects that vary from their individual exposure. Hence resulting in the changed absorption via the BBB (Sanders et al., 2015). It has been observed that instead of isolated metals, the mixture of metals has rather much more capability of causing general outcomes e.g. dysfunction of cognition (Basha et al., 2005).

Considering the reference of certain studies it is concluded that the mixture of Pb and Cd can have greater impacts than additive consequence on the synthesis of DMT1 in the evolving rat brain, hence leading to increased rate of metals transport and results in greater cognitive dysfunction (Karri et al., 2016). Furthermore, results from the experimentation on expecting rat suggested that the co-exposure of Cd & Pb have combine effect on sodium potassium ATPase pump (Na+/K+ ATPase) pump and decreased the activity of Na+/K+ transporter. Cd combine with Pb which can disturbed the function of Na+/K+ pump. Due to inhibition of the transporter, there is reduction of K+ ions inside the cell and excess of Na+ ions and calcium ion resulting in enhanced cognitive deficits (Antonio et al., 2003). Likewise, there has been another study of binary mixture of Pb and As in nervous system,

suggesting that the co-exposure of metals affects the hippocampus by significantly intensified action of Pb in the existence of As (Tyler and Allan, 2014).

It has been observed experimentally that the ternary exposure of As, Cd & Pb results in the activation of intracellular Ca+2 followed by ROS production, stimulates complex pathways like c-Jun N-terminal kinases (JNK) pathway, mitogen-activated protein kinase 3 (MAPK3) leading to oxidative damage in the neurons. Despite this, it resulted to be unsuccessful in order to explain strong epidemiological connection within ternary mixture (Pb, As, and Cd) contact and cognitive disabilities (Guilarte et al., 2000).

Considering the research, it can be said that much greater impacts of a mixture of Pb, Cd, As and MeHg which generates general adverse consequence called as cognitive dysfunction or dementia via triggering the various interactions in hippocampus. The true toxicological processes craved by exposure to mixtures of the metals are still not explained clearly. In addition to that it was figured that every metal share similar cellular properties like receptors, protein composition, neurotransmitters for influencing cognition (Guilarte et al., 2000).Thus, it can be considered that the mechanism of toxicity of individual metal can be utilized as a standard for comprehending possible mechanisms related with mixture (Pb, Cd, As, and Al). In addition hypothesis can be made that co exposure to Pb, Al, As have much greater than toxic responses resulting in neuroinflammation. Mixture of metals have greater absorbance capacity to BBB rather than single metal, for this reason combined exposure causing greater toxicity to the CNS (Schwartz et al., 2005).

### 1.4 High Fat Diet (HFD) Consumption

HFD is composed of unsaturated and saturated fats. Amount of fat in the diet are important for maintaining balance in energy. Intake of high fat diet are the main cause for endemic. Diet which are rich in greater than 30 % fat are the main cause of obesity (Pistell et al., 2010). Ingestion of HFD is one of the major risk for obesity. Obesity itself represent low grade inflammation and it is the prime cause of many diseases including cardiovascular diseases, metabolic disorders and cancers. Studies were carried out and it was clearly observed in rodents and humans, that obesity is linked with learning and memory impairement which in turn leads to Alzheimer's disease (Winocur et al., 2005) and alters normal body functioning, burdens the brain and effect the normal physiological functions of the nervous system that openly influence the hippocampus which is important for learning and memory (Furukawa et al., 2017).

HFD altered glucose regulation which leads to less production of growth factors, lower expression of the tight junction proteins and increased permeability of the BBB. This effects the structure and function of the BBB and caused neuroinflammation (Hu et al., 2001). It upregulates the level of cytokines (IL 1β, IL 6, TNF α) and other inflammatory mediators which leads to the activation of complex inflammatory pathways (Pistell et al., 2010). High fat diet leads to the production of ROS which represents oxidative stress in different brain regions like cortex and specially hippocampus that results in inflammation which adversely affects cognition. Many studies have conducted that proposed connection in the pathogenesis of Alzheimer's disease and high fat diet consumption (Hsu and Kanoski, 2014). Hippocampus, the main part of the nervous system which is vulnerable to diet content especially high fat diet and have a key role in neurogenesis (Tozuka et al.,

2009). Studies has demonstrated that HFD have the potential to disrupt synapse formation and synaptic activity (Farr et al., 2008).

### 1.4.1. Effect of High Fat Diet on CNS

High fat diet indirectly alters the normal functioning of the nervous system and affect learning and memory. Excessive HFD intake results in increased accumulation of fatty acids in adipose tissues due to which there is upregulation of immune cells in adipose tissue. In the systemic circulation, the level of inflammatory cytokines and other mediators like LPS (lipopolysaccharide) and FA (fatty acid) are high, due to which an inflammatory response is generated in the periphery (Hou et al., 2019).

There are different pathways by which CNS can be influenced i-e through choroid plexus, vagus nerve and the barrier present between the nervous system and the periphery the blood brain barrier (Hou et al., 2019). The inflammatory signal which is generated in the periphery due to increased inflammatory mediators passed to CNS through vagus nerve. The inflammatory mediators get access to the BBB due to which BBB permeability increases and resident immune cells get access from the periphery to the CNS (Louveau et al., 2015). Neuroinflammation occurs due to astrocytosis and gliosis which represents upregulation of inflammatory cytokines and other mediators in the CNS. Activated microglial cells interferes with neurons and damage the structure of neurons (Guillemot-Legris and Muccioli, 2017). HFD ingestion is associated with higher body mass Index (BMI) and it is one of the risk factor for neurodegenerative diseases like Alzheimer's disease and some form of memory loss (Anstey et al., 2011). Some structural modifications occurs in the hippocampus due to which the integrity of the cerebrum decreases (Elias et al., 2005). It also leads to cognitive dysfunctions in which a person experience poor

attention, difficulty in speaking, reasoning, making decisions, learning and memory deficits (Kramer et al., 2007).

### 1.5 Effect of Heavy Metals and HFD on BBB

Blood brain barrier (BBB) is composed of specific vascular structure. It is comprised of endothelial cells (ECs) that form the boundary of the blood vessels and they are joined together by tight junctions, basement membrane and some glial extensions (Rubin and Staddon, 1999). ECs of nervous system has specific properties as compared to ECs of other tissues. ECs of BBB has special transporters for exchange of ions, nutrients and removal of toxic compounds from CNS to periphery. BBB is a physical barrier between the blood and CNS and this limiting barrier tightly regulate the normal homeostasis of the nervous system. During normal physiological condition, BBB has less penetrability but in case of infection, injury or any toxins the permeability of the BBB increases followed by transport of ions other mediators into the brain (KrolI et al., 1998).

For proper functioning of CNS, there must be an optimum amount of essential metals (Ca, Mn, Co, Zn, Cu, Mg) delivered to the nervous system because in case of insufficiency and surplus amount of metals cause abnormal function of the nervous system. There are specific metals transporters, DMT1 and Ferroportin (iron exporter) which is present across the membrane and helps in an iron transport across the BBB and keeping the optimum level of metals (El-Masri, 2007). Non-essential metals like Pb, Hg, As, Al accumulated in the body can also get entrance to the BBB and accumulated at the tight junction and finally disrupt the structure & function of the barrier ( Figure 1.3) (Zheng, 2001). Pb toxic effects on the CNS are well known. Pb competes with iron at the junction of the BBB and competitively inhibit iron which affect calcium signaling, change the structure of protein

and hinders BBB functions. Consequences of Pb toxicity are nervous system disorders, organ damage, accumulation of CSF in ventricles and breathlessness etc. Choroid plexus is more vulnerable to lead than endothelial cells of the BBB (Friedheim et al., 1983). Cohort studies were conducted using animal models and it is demonstrated that HFD (unsaturated fatty acids/western diet) ingestion disrupt the BBB and cause neuroinflammation. It reduces the expression of tight junction proteins (occludin) that results in impairment of the BBB and cause dysfunction of different brain regions especially hippocampus (Davidson et al., 2012).

## 1.6 INF LAMMATORY MARKERS

### 1.6.1 Tumor Necrosis Factor Alpha (TNFα)

Tumor necrosis factor (TNF α), which is also called cachectin. It is composed of 233 amino acid located at chromosome 6p21.33. O-glycosylation occurs during post translation modification of TNF α. It is a widespread cytokine which have a key role in many processes like cell death, cell division, blood clotting, cell differentiation, metabolism of lipids, various types of cancers and insulin resistance. During physiological condition of a body TNFα levels are high in the systemic circulation and acts as inflammatory biomarker (Holmstrup et al., 2017). During normal body condition, observed level of TNFα are 4–11 pg/ml in human serum (Zhu et al., 2016) and it have a neuroprotective effect as it sense the inflammatory mediators in the systemic circulation, clear the infection and induce cell repair at that site (Holmstrup et al., 2017). It was observed when mice model exposed to to metals (Al, As) for 5 months the level of TNF α was increased in the systemic circulation (Dong et al., 2015). Microglial cells which are the resident immune cells of the CNS, depends on TNFα to perform its role in cell death and inflammatory pathways.

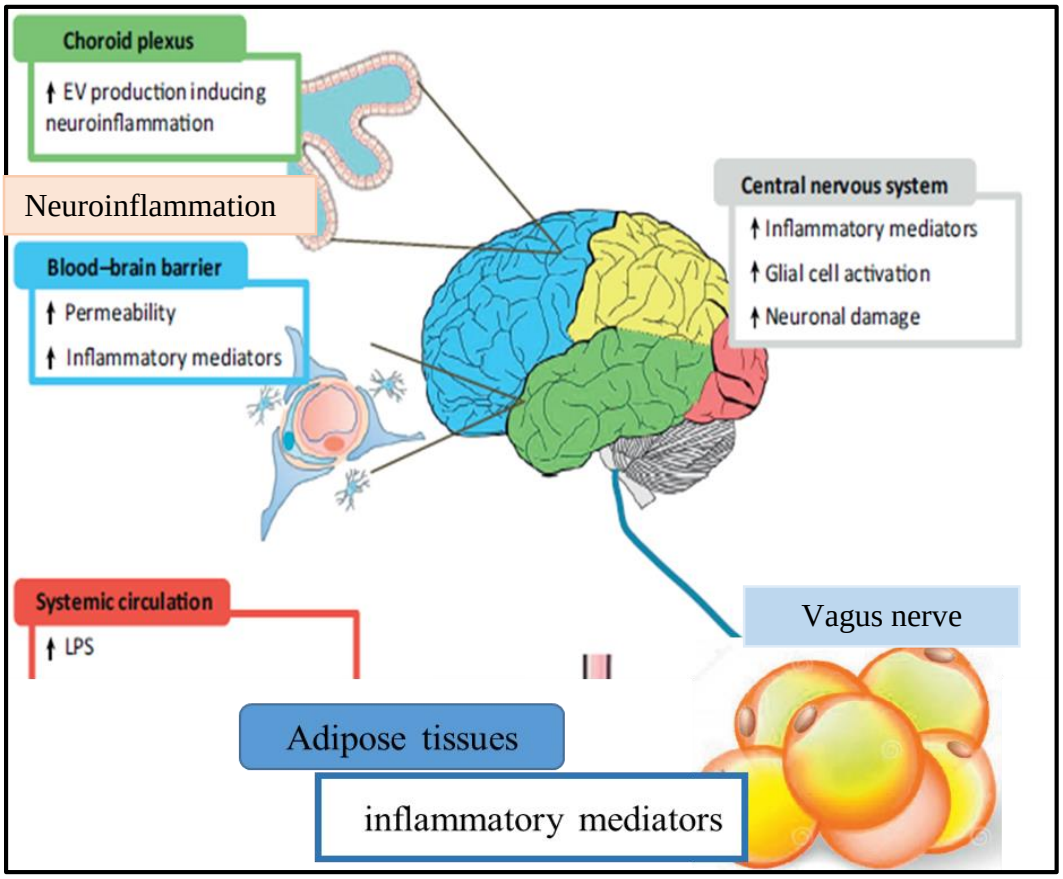



**Figure 1.3 Effect of HFD on the central nervous system:** HFD induces inflammation throughout the body. LPS (lipopolysaccharide). Adapted from (Liu et al., 2019).

Proinflammatory mediators along with TNF α is released during pathological condition. These inflammatory cytokines i-e TNFα and IL-1β impairs BBB, increased the expression of adhesion proteins and increase the production of toxic compounds that interferes with calcium signaling (Lyman et al., 2014). The neurons and astrocytes both have potential to synthesize TNF-α, in case of inflammation glial cells produces inflammatory cytokines and mediators. Interferon gamma (IFN-γ) which is a cytokine and stimulator of glial cells which in turn up regulate expression of TNF-α and involved in many complex inflammatory cascade and signaling pathways like c-JUNK ,MAPK, NF-κB, p38 and ERK1/2 (Olmos and Lladó, 2014).

### 1.6.2 Glial Fibrillary Acidic Protein (GFAP)

Glial Fibrillary Acidic Protein which is an astrocytic marker and greatly expressed in the nervous system (Zhang et al., 2014). Due to its abundance, it is also present outside the nervous system in the peripheral extensions (sciatic nerves) of peripheral nervous system. GFAP is confined to non- myelinated Schwann cells which perform same function like astrocytes. The cells which expressed GFAP on their surfaces have some connections with the gut region through enteric nervous system that innervated their neurons at the sub mucosal layer and execute its function like the uptake and degradation of neurotransmitter by glial cells to modulate the neurons (Mokuno et al., 1989).

During pathological condition or any injury to the CNS, astrocytes and glial cells are activated, the normal physiology changes from normal to abnormal which represents astrocytosis and microgliosis. GFAP levels are also up regulated in the CNS in case of neuroinflammation which represents astrogliosis in which astrocytes and microglial cells are in activated state and proliferate (Eng et al., 2000). During neuroinflammation, changed

expression of many proteins, genes and inflammatory mediators are observed. (Orre et al., 2014).

### 1.6.3 Interleukin-1 Beta (IL1 β)

Interleukin1β is pro-inflammatory cytokine present in cytosol and it is synthesized by myeloid cells (MC), inflammatory mediators, resident immune cells of the CNS (astrocytes and microglial cells) during disease pathogenesis (Dinarello, 2011). IL1β is first synthesized in inactive form (immature IL1β) which is converted into active (mature IL1 β) form by the cleavage of caspase-1. It shows pleiotropism which means having multiple functions in the CNS that includes maturation, separation and proliferation of neurons, cognitive functions, cell death and apoptosis. Its role in inducing neuroinflammation has been proven in many diseases which includes different autoimmune diseases, different neurodegenerative disorders like multiple sclerosis, Alzheimer's disease, TBI (Traumatic brain injury) and stroke (Mendiola and Cardona, 2018).

IL1 β secretion in hippocampus is associated with learning and memory. It helps in memory consolidation. Cohort studies were conducted on IL1β deficient mice model, which showed impaired learning & memory process. When small amount of interleukin1β administered to these mice model, it recovered normal memory formation in the hippocampus. Hence, it is proved that IL-1 signaling cascade play a key role in proper functioning of the hippocampus. But greater secretion of IL-1 β in the CNS have negative effect on memory formation. From all these facts, it is clear that the normal physiological level of IL1β affect hippocampus (Trenova et al., 2016).

### 1.7 Natural Products as Inhibitors of Neuroinflammation

Different plants and their active constituents have been used for many decades .They are tested time to time and validated in various experimental studies. The herbal medicines are traditionally used for many purposes. These natural products having pharmacological properties i-e neuroprotective, anti-oxidant, anticarcinogenic, anti-inflammatory and have potential to control cell damage and neuronal death. The active ingredients of herbal medicines have the ability to block or inhibit various neuroinflammatory mediators to protect brain from neuroinflammation, to reduce neurodegeneration and learning and memory impairment up to some extent. The active constituents of natural compounds acts through different mechanisms, followed specific pathway of neuroinflammation and have ability to inhibit microglial cells and might be effective in improving neurodegenerative and neuroinflammatory diseases that is associated with activated microglial cells (Chen et al., 2007).

#### 1.7.1 Therapeutic Potential of Ginger

Ginger, belong to the family Zingiberaceae is the root of Zingiber officinale. It is used traditionally as a flavoring agent in cooking and for cure purposes. The active constituents of ginger are oleoresin and terpenes which comprised ginger oil. Ginger consists of volatile oils 1-3% and the pungent constituents which is non-volatile called oleoresin. The major characterize components of ginger are phenolic compounds and organic compounds which are Shogaol and gingerol (Jolad et al., 2004). Ginger extract produced active compound like 6-Shogaols, 6-gingerol, paradol, zingerone etc. 6-gingerol being the most abundant component in the rhizome of ginger. Ginger have been used as neuroprotective, anti-inflammatory, anti-oxidant, anti-tumorigenic agent. It is reported that ginger and its active

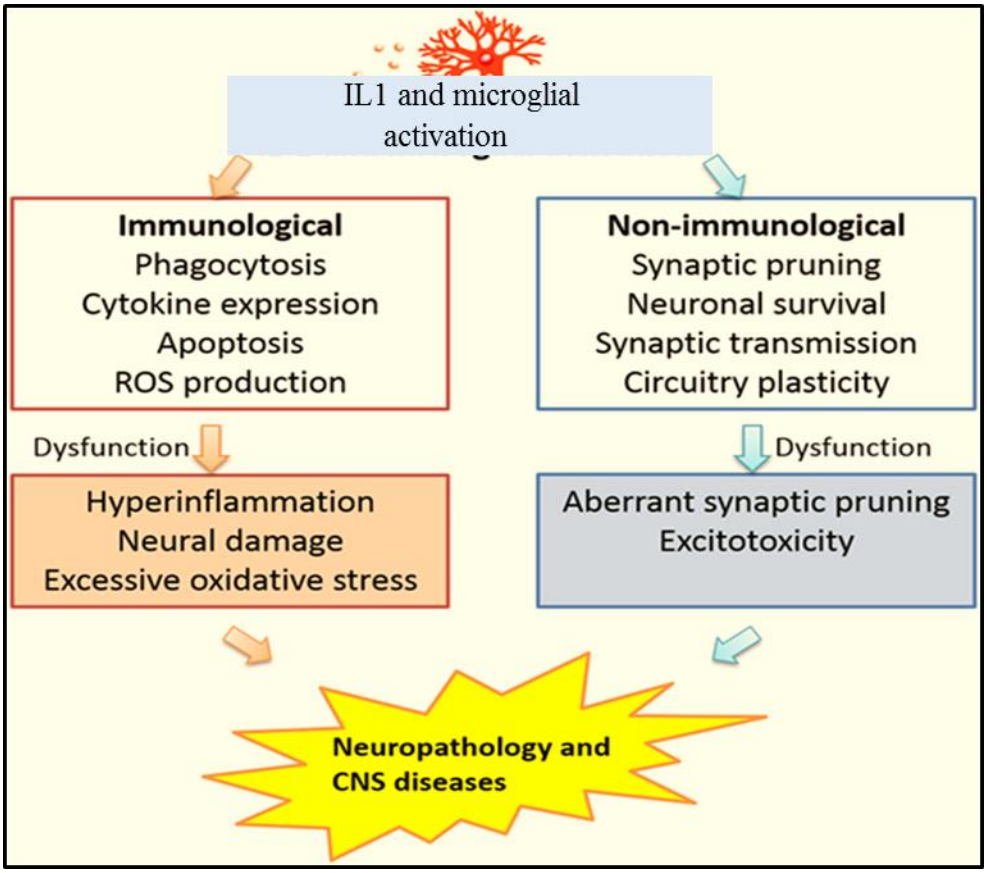



**Figure1.4** Immunological and non-immunological aspects of IL1β.Adapted from (Liu and Quan, 2018).

constituents show positive effect on learning and memory (Saenghong et al., 2012).

### 1.7.2 Shogaol

Shogaol is formed by the dehydration of gingerol. It is the main active constituent of ginger. They are present in less amount in freshly prepared root but greater quantity of Shogaol are present in dehydrated or heat treated rhizome (Jolad et al., 2004). Shogaol, the phenolic component of ginger and it have many properties. 6-Shogaol have anti-inflammatory, anti-carcinogenic, antipyretic, antitussive, antioxidant, neuroprotective effects (Ha et al., 2012). Researches have been carried out which confirmed that Shogaol have potential to block inflammation which is a complex process that involves various inflammatory mediators and cytokines IL-1β, TNF-α, GFAP etc. Nowadays different antagonist are used for inhibiting inflammation but it poses negative side effects on the body. To overcome this problem, different natural compounds and their active components play a key role in the inhibition of neuroinflammation and demonstrated anti-inflammatory effect. Moreover, Shogaol employs its effect by blocking activated microglial cells and astrocytes. It decreases cognitive impairment by blocking different inflammatory pathways (MAPK, COX-2, NF-κB). When Shogaol is administered to memory impaired mice model, considerable improvement in learning and memory is observed (Moon et al., 2014)

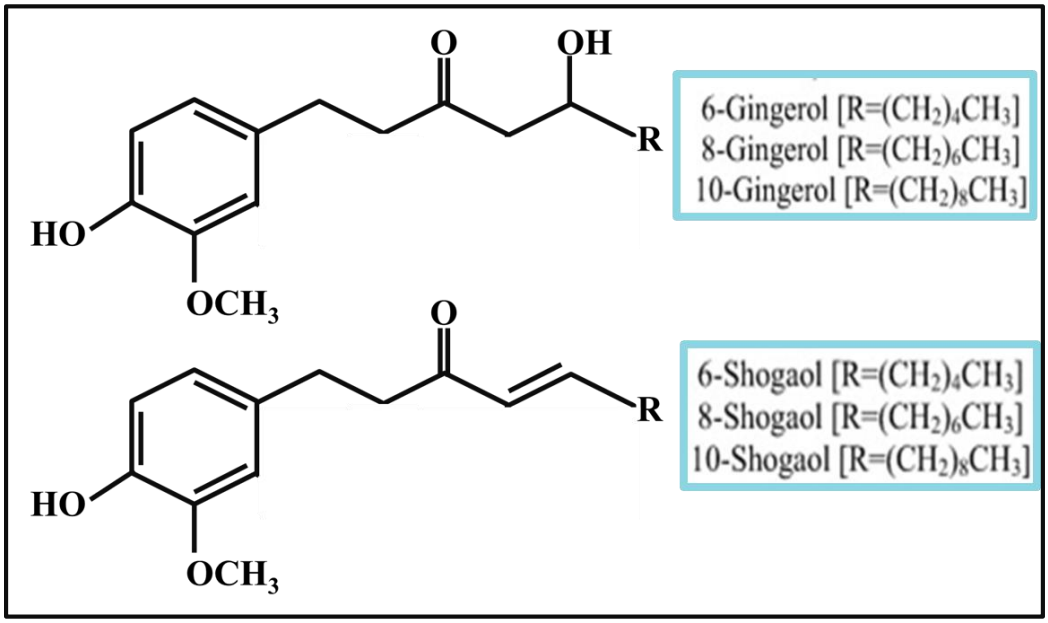



**Figure 1.5 Chemical of structure of active constituent of Gingerol and Shogaol.** Adapted from (Wu et al., 2010)

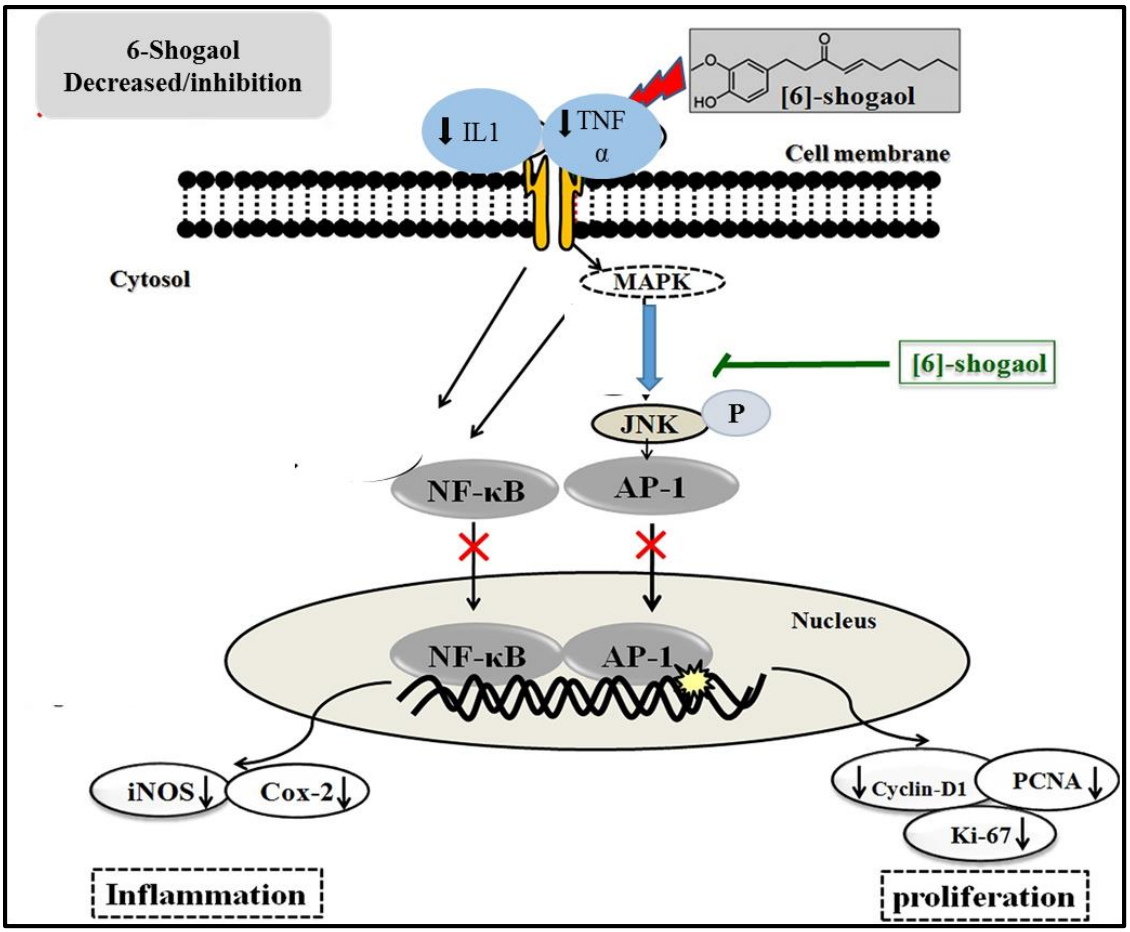



**Figure 1.6 Pathway of the molecular mechanism of 6-Shogaol to inhibit inflammation**: Nuclear factor kappa B (NF- κB), tumor necrosis factor (TNF-α), interleukin 1 (IL-1), Jun N- terminal kinase (JNK), activator protein (AP-1), inducible nitric oxide synthase (iNOS),Cyclooxygenase-2 (Cox-2), proliferating cell nuclear antigen (PCNA) have a role in initiating the anti-inflammatory effect of Shogaol. Adapted from (Annamalai and Suresh, 2018).

## 1.8 Research Objectives

1. To evaluate the effect of Shogaol on the blood brain barrier in metals+HFD induced mice model.
2. To investigate the effect of Shogaol on the gene expression level of inflammatory markers IL-1 beta, TNF-α and GFAP in the cortex and hippocampus of high fat diet and metals induced mice model through real-time polymerase chain reaction (qRT-PCR).

***Chapter 2***

# MATERIALS AND METHODS

## 2.1 Ethics Statement

All the experiments were carried out under the approval of Internal Review Board ASAB, NUST. The protocols for this experiment were performed according to the guidelines of Institute of Laboratory of Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA (Guide for the Care and Use of Laboratory Animals, Eighth Edition, 2011).

## 2.2 Chemicals and Reagents

Chemicals such as Aluminum chloride hexahydrate AlCl3·6H2O (AL0770), was bought from Scharlau, Spain. Impurities content of AlCl3· 6H2O was less than 0.005% of the total impurities of heavy metals. Sodium arsenate Na3AsO4 (A6756) and Lead acetate trihydrate Pb (CH3COO) 2.3H2O (11504) were purchased from Sigma -Aldrich, Germany. Reagents like Taq Polymerase, Revert Aid Reverse Transcriptase, Deoxyribonucleotide triphosphate (dNTPs), oligo-dT, were purchased from Fermentas (Thermo Scientific, USA). Trizol reagent and SYBR Green dye were obtained from Invitrogen (Thermo Scientific, USA). All the other chemicals used in this research were obtained from Sigma-Aldrich (USA).

## 2.3 Animals

Experiments were carried out on male Balb/c mice (8 weeks old), purchased from National Institute of Health, Islamabad, Pakistan and kept in Laboratory Animal House of Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST. The animals (n=44) were then

kept in regular cages which were cleaned and placed under the standard conditions of living at a constant temperature of 25 ± 2 °C with a 12 hours light/dark cycle. They were provided with standard feed. All the animals were divided into groups and housed in the similar cages and had free access to feed and water.

**2.4 Study Design**

Animals were divided into four experimental group, each group consisting of 11 mice. Each metal (Al, As, Pb) were given orally mixed with distilled water. These metals were given at the concentration of 25mg/kg of the body weight of mice along with HFD (40%) in normal feed (60%) were provided to mice for 8 weeks (60 days) to develop chronic mice model of metals and HFD. As this was a preventive study, Shogaol (treatment compound) was given at the same time with metals and HFD. Distilled water and basic mice feed was provided to control group. The animals (n=44) were divided into four groups of 11, each *[Table 2.1]*

**Table 2.1** Study groups division

| S.no | Groups | Description | No of mice |
|---|---|---|---|
| **1** | Control | Distilled water + basic mice feed | 11 |
| **2** | Diseased | Metals in water + HFD | 11 |
| **3** | Low dose treated | Heavy metal + HFD + Shogaol 2mg/kg | 11 |
| **4** | High dose treated | Heavy metal + HFD + Shogaol 12mg/kg | 11 |
| **Total Number of mice** | | | 44 |

**HFD= High fat diet**

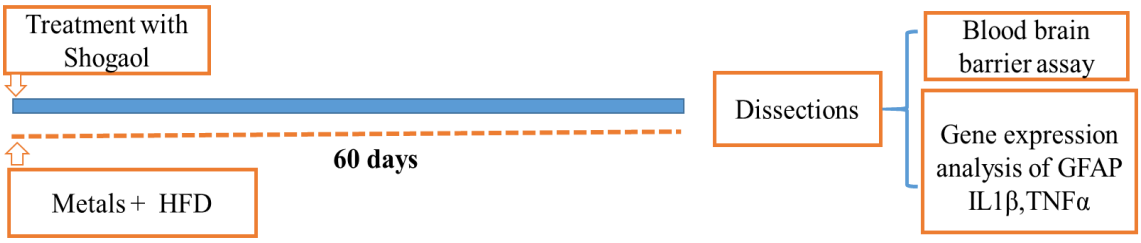



**Figure 2.1** Study design

### 2.5 Mouse Decapitation and Brain Harvesting

Mice were anesthesized using chloroform. After few seconds the head of mice was insistently extended from the anterior side. By using surgical scissors a cut was made at the neck region which separates head from the rest of the body. After decapitation skin of the head region was removed to expose the skull. Incision was made at the caudal part which was extended to the frontal part of the skull. When the skull was opened, whole brain was clearly seen. The brain was lift through forceps from the skull to petri plate wrapped with aluminium foil that was pre chilled. Cortex and hippocampus were isolated using forceps and used for BBB assay and RNA extraction.

### 2.6 Blood Brain Barrier Assay

For checking permeability of the BBB, freshly prepared Evans Blue dye (2% solution) was made in normal saline. EB dye (4 ml/kg) of the total body weight of mice was administered intraperitoneal. After dye injection, it was allowable to flow in the blood for 3 h. After 3 h, mice were perfused with 50ml of chilled PBS through left ventricle at 120 mm pressure. Afterward the brain was exposed and transferred to pre chilled petri plate covered with aluminium foil. The brain was divided into right and left hemisphere, 20 mg of brain tissue was taken from both the hemispheres and quickly snap frozen in liquid nitrogen to avoid degradation.1100 µl of PBS was added to both the samples of right & left hemisphere and were then homogenized, sonicated, centrifuged at 15,000 rcf for 30 min at 4°C. Two separate layers can be seen after centrifugation, colorless upper layer approximately 500µl was transferred to new tubes for further processing and the dense lower layer was removed.

Then 500µl of 50% trichloroacetic acid (TCA) added to each sample and incubated the samples for 24h at 4 °C. Next day the samples were again centrifuged at 15,000 rcf for 30 min at 4 °C. Different dilutions of 500 µg, 100 µg, 20 µg, 4 µg, 0.8 µg of EB dye were made. Optical density of the dilutions and samples were measured by spectrophotometer at 610 nm and was calculated according to the standard curve. Results are represented as (µg of Evans Blue stain) / (mg of tissue) (Manaenko et al., 2011).

## 2.7 GENE EXPRESSION ANALYSIS

### 2.7.1 RNA Extraction

RNA extraction was done according to published protocol using Tri-reagent. Cortex and hippocampus isolated from the brain was washed with phospate buffer saline (PBS) Trizol (1 ml) added to each sample to maintain RNA integrity, then the samples were sonicated to homogenized brain tissues in Ultrasonic Sonicator.The samples were then incubated at room temperature for 10 min followed by the addition of chloroform (200µl) to each sample and instantly mixed well for few seconds. Again the samples were kept for 15 min at room temperature,then the samples were centrifuged at 12,000 rpm for 20 min at 4˚C Two layers can seen after centrifugation,upper aqueous layer was transferred to new tubes and the lower viscuous layer was dicarded. Isopropanol 500 µl was added to further separate the layers, followed by incubation of 15 min and then the samples were centrifuged at 12,000 revolution per minute (rpm) for 15 min at 4˚C After centrifugation, a pellet was seen in the bottom of the eppendorf tube and RNA was precipitated and the supernatant was removed and discarded. The pellets were washed with 80% ethanol (1 ml)

and finally centrifuged at 75000 rpm for 5 min at 4˚C. RNA pellets were resuspended in nuclease free water NF (30 µl).

### 2.7.2 Quality of RNA

Before complementary DNA (cDNA) synthesis all the RNA samples of cortex and hippocampus were run on 1% agarose gel for 40 min which was then visualized on Gel Doc (Wealtec Bioscience Co., USA) to confirm the quality of RNA and to ensure that the RNA is pure and reproducible to be used further for cDNA synthesis.

### 2.7.3 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

For cDNA synthesis, RNA samples (cortex + hippocampus) were quantified using the following ingredients. The reaction mixture contain 2 µg RNA, 10 mM dNTP's, 5mM oligo-dT, 8 µl of RT buffer, 1 µl of RNaseout$^{TM}$ Ribonuclease inhibitor, 2 µl of Revert aid RT and finally PCR water to make the finishing volume up to 25 µl. The microfuge tubes contain the reaction mix was homogenized using vortex. The temperature cycle conditions for cDNA (complementary DNA) synthesis was 42°C for 60 min and 70°C for 10 min (*Figure 2.2*). Synthesized cDNA was kept at -80$^{o}$C for further use.

### 2.7.4 cDNA Amplification

cDNA was amplified using GAPDH primer through conventional PCR.The reaction mixture contain 2 µl cDNA, 2.5 µl 10X Taq buffer, 1 µl 10mM, dNTPs mix, 2.5 µl 25mM, 2.5 µl $MgCl_2$,1 µl forward and reverse primers, 0.5 µl Taq polymerase and finally added 15.5 µl PCR water to make final volume up to 25 µl. The product size was analyzed using 2 % gel, clear bands were seen which confirmed that cDNA can be used for real time PCR.

Primers were also optimized using conventional PCR. Temperature profile for cDNA amplication is given in (*Figure 2.3)*

### 2.7.5 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Real time PCR were carried out for the gene expression analysis.The reaction mix consists of 1µl of forward & reverse primers,1 µl cDNA and 4 µl of Thermofischer scientific SYBR green ,all the mixture were mixed up through vortex and amplified according to the cycling profile as given in (*Figure 2.4).* Using specific forward and reverse primers [*Table 2.2*] the expression of GAPDH, IL-1 beta, TNF-α and GFAP was analyzed using ABI Prism 7300 sequence detection system (Applied Biosystems, 7300).

For checking the reliability of PCR products, 2 µl of PCR product mixed with 5 µl of loading dye, poured in the wells of gel and were then run on 2% gel, later on visualized by gel Doc. The dissociation curve and amplication plots were also analyzed.The values for GAPDH was obtained which acts as a control and all the other values of genes were normalized to the value of GAPDH which was then calculated using 2- ΔΔCT method.

- ΔCt (Control) = Average Ct of target gene (control) – Average Ct of GAPDH (control)
- ΔCt (Target gene) = Average Ct of target gene (Exp.) – Average Ct of GAPDH (Exp.)
- ΔΔCt= ΔCt (target gene) – ΔCt (control)
- Fold change= 2- ΔΔCT

### 2.8 Statistical Analysis

All the data were analyzed by one-way analysis of variance (ANOVA) which is followed by column & grouped statistics. Bonferroni multiple comparison tests was applied to determine the difference between each group. The results were expressed as the mean ±standard error of the mean (S.E.M.). The results were significant when $p < 0.05$. The graphical data was generated using the Graph Pad Prism 5 software.

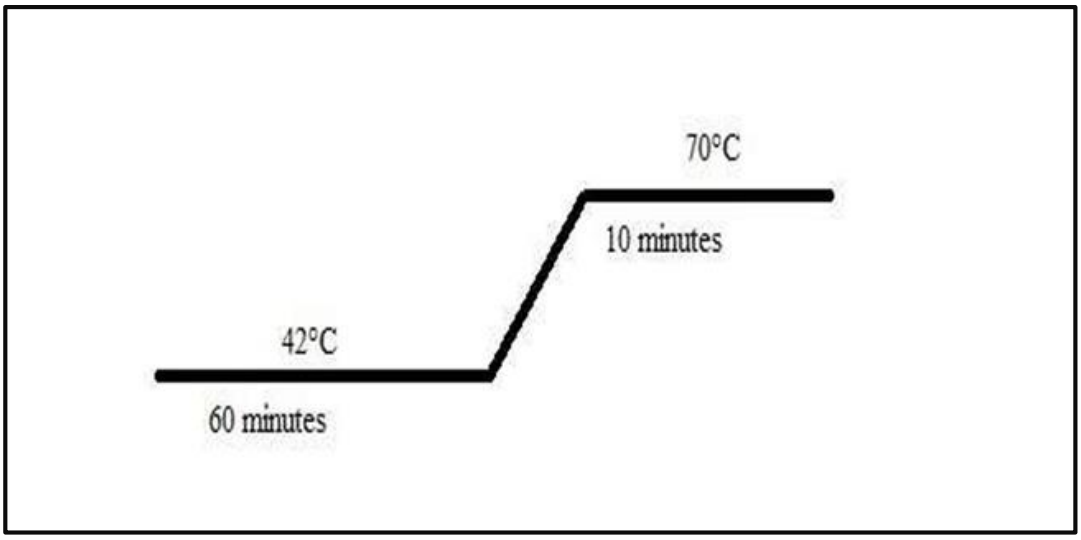



**Figure 2.2** Temperature pofile for cDNA synthesis

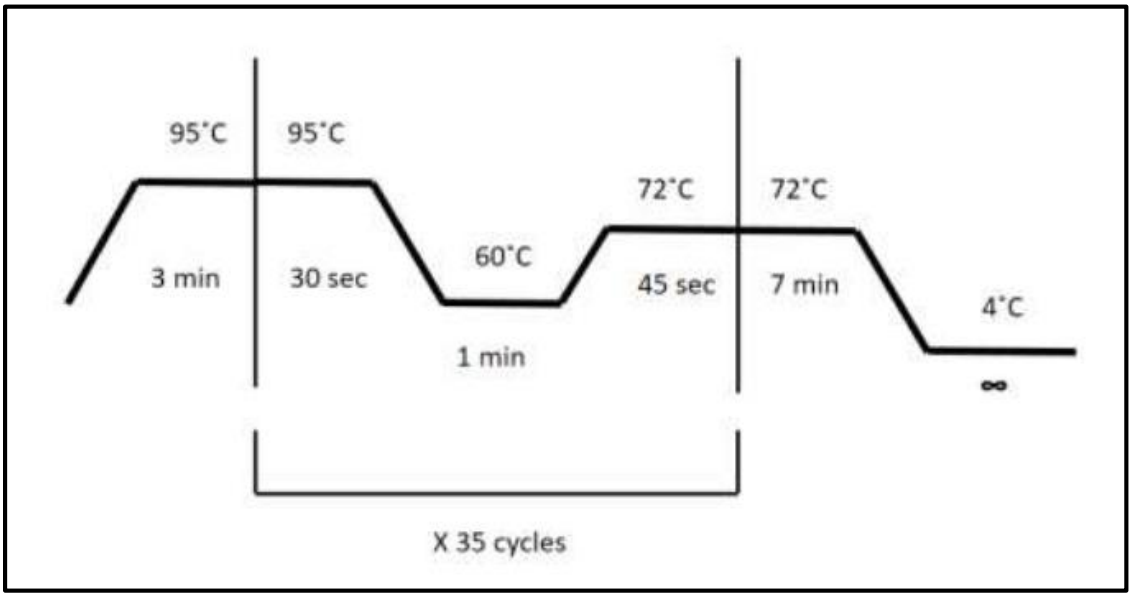



**Figure 2.3** Temperature pofile for cDNA amplification

**Table 2.2** Real-time PCR primer sequences (mouse) used in the study

| **Gene** | **Primer** | **Sequence 5' – 3'** | **AT** |
|---|---|---|---|
| **IL-1 beta** | Forward | TGAAGAAGAGCCCATCCTCTG | 60ºC |
| | Reverse | GGGTGTGCCGTCTTTCATTA | |
| **TNF-α** | Forward | ATGAGCACAGAAAGCATGA | 62ºC |
| | Reverse | ACCACGCTCTTCTGTCTACT | |
| **GFAP** | Forward | TGCAAGAGACAGAGGAGTGG | 60ºC |
| | Reverse | GCTCTAGGGACTCGTTCGTG | |
| **GAPDH** | Forward | ACCCAGAAGACTGTGGATGG | 60ºC |
| | Reverse | CACATTGGGGGTAGGAACAC | |

Interleukin-1 beta (IL-1β), tumor necrosis factor (TNF-α), glial fibrillary acidic protein (GFAP), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), annealing temperature (AT).

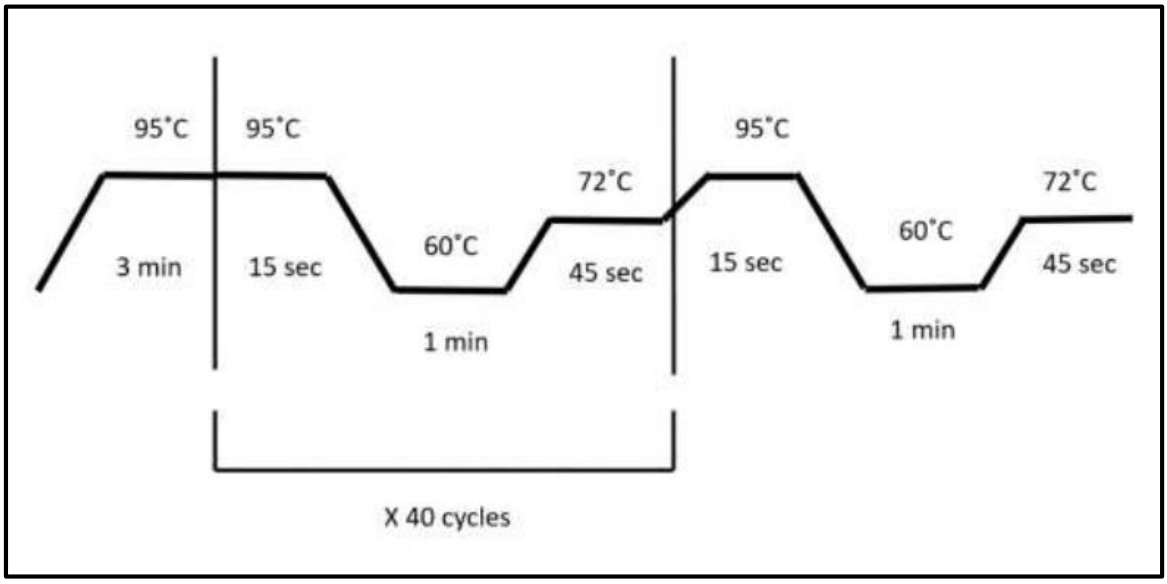



**Figure 2.4** Real time PCR Profile: Step 1 denaturation or holding step, Step 2, annealing/extension step, Step 3, dissociation step.

***Chapter 3***

# RESULTS

## 3.1 Blood Brain Barrier Assay

### 3.1.1 Evans Blue Quantification

Different concentration of Evans blue bye were taken and plotted to obtained a standard curve at 620nm. Different optimal density (OD) showed by each concentration of Evans blue dye. Sample absorbance was also measured at 620nm and then compared with the OD of dilutions of standard concentrations of Evans blue to find the concentration of Evans blue in the sample.

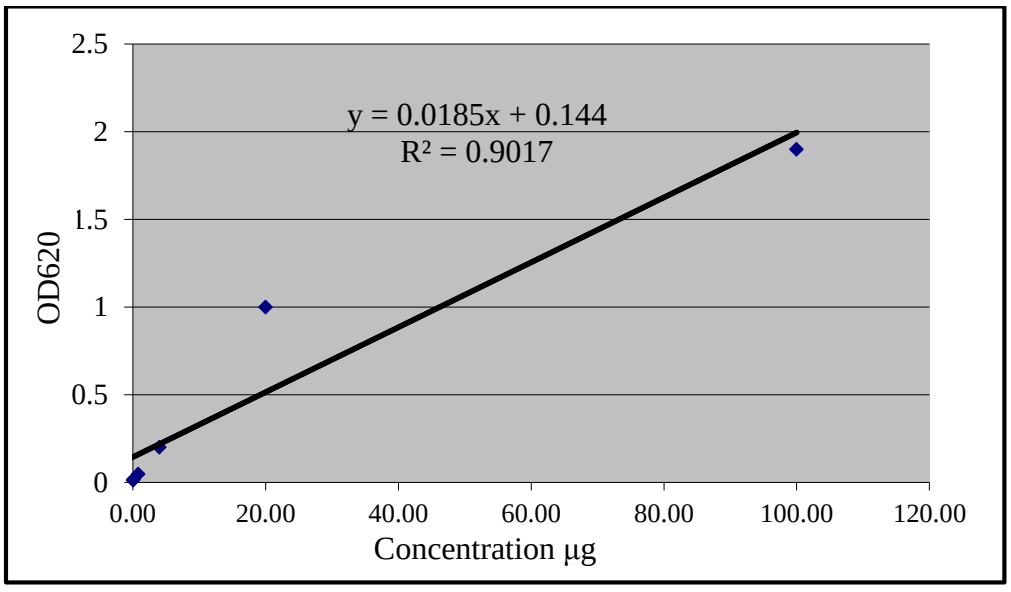



**Figure 3.1 Standard curve for various concentration of Evans blue dye:** Concentration was plotted on the x-axis and optical density (absorbance at 620nm) was plotted at y-axis. Linear regression value was 0.9017 ($R^2$=0.9017).

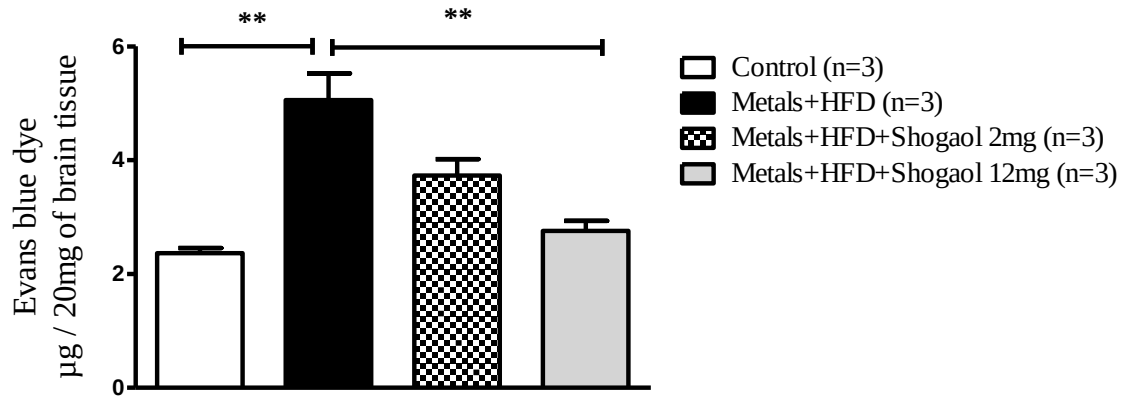
### 3.1.2 Evans Blue Extravasation in Right Hemisphere

The blood brain barrier assay was carried out to check the leakage in mice brain tissue (Right and left hemisphere). Blue discoloration was observed in mice brains which clearly shows the tissue leakage. In the right hemisphere *(Figure 3.2 A)* it was observed that as compared to control (2.3 ± 0.08), a significant increase ($p<0.01$) in the amount of Evans blue dye in the right hemisphere of mice fed with metals+HFD (5.05 ± 0.46) for 8 weeks Shogaol normalized ($p<0.01$) the amount of EB dye in metals+HFD+Shogaol 12mg group (2.75 ± 0.17) as compared to metals+HFD (2.36 ± 0.08). While there was no significant difference between metals+HFD group and metals+HFD+Shogaol 2mg group. Similarly the extravasation of EB dye did not vary significantly between control and metals+HFD +Shogaol 2mg and 12 mg groups *(Figure 3.2 A).*

### 3.1.3 Evans Blue Extravasation in Left Hemisphere

In the left hemisphere region *(Figure 3.2 B)* of mice brain, it was observed that in comparison to control group (1.52 ± 0.03), a significant extravasation in the amount of EB dye ($p<0.05$) was observed in metals+HFD (1.98 ± 0.11) group as compared to control. When Shogaol was administered, there was a non-significant trend observed in metals+HFD+Shogaol 2mg group compared to metals+HFD group. In comparison with metals+HFD (1.98 ± 0.11) group, a significant decrease ($p<0.01$) in the amount of EB dye was observed in metals+HFD+Shogaol 12mg group (1.40 ± 0.05) *(Figure 3.2B).*

**A** Right hemisphere




**B** Left hemisphere

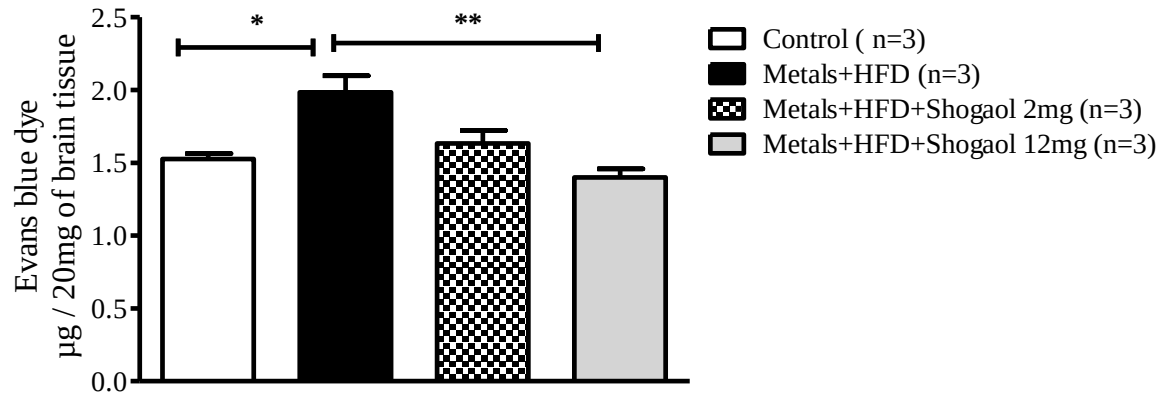



**Figure 3.2 Permeability of the blood brain barrier using Evans blue dye in right hemisphere (A) and left hemisphere (B)**. The graphs show the leakage of the blood brain barrier in control, metals+HFD, metals+HFD+Shogaol 2mg and metals+HFD+Shogaol 12 mg groups. Data were analyzed using one way ANOVA followed by Bonferroni multiple comparison test and are displayed as mean ± S.E.M **p=0.01 *p=0.05.

## 3.2 GENE EXPRESSION ANALYSIS

### 3.2.1 RNA Confirmation

Extracted RNA of all groups' i-e control, metals+HFD, metals+HFD+Shogaol 2mg and metals+HFD+Shogaol 12mg was confirmed using 1% agarose gel. Sharp and clear band of 28S & 18S subunits was seen on the gel which confirmed good quality of RNA as shown in *(Figure 3.3).*

### 3.2.2 Complementary DNA (cDNA) Confirmation

All the cDNA products were amplified with GAPDH having product size of 175bp. Amplification product of all groups control, metals+HFD, metals+HFD+Shogaol 2mg and metals+HFD+Shogaol 12mg were run on 2% gel to confirmed the synthesized cDNA. Very clear bands at 175bp position was seen as shown in *(Figure* 3.4) which indicates that cDNA can be further used in quantitative real time PCR for transcriptional analysis.

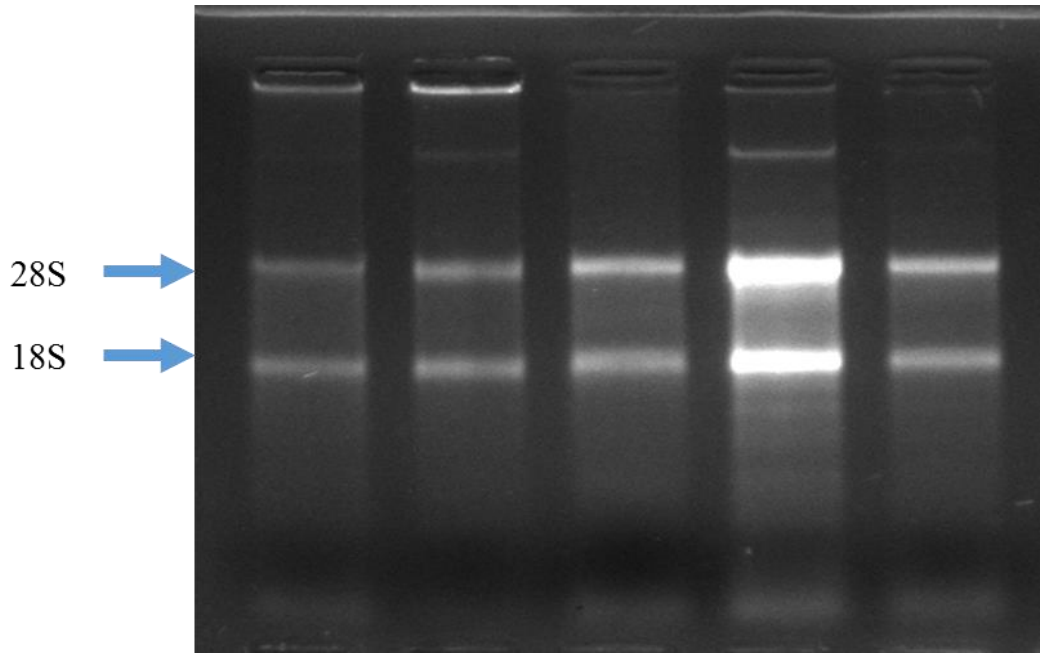



**Figure 3.3** Gel image of clear bands of 18S and 28S subunits of RNA to confirm RNA quality

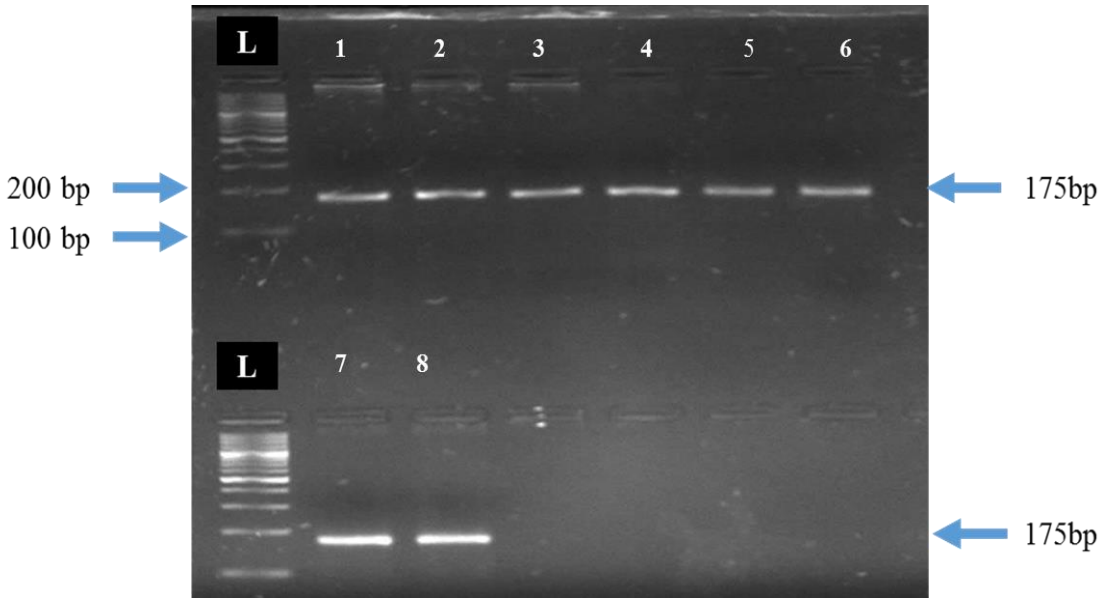



**Figure 3.4** Gel image of cDNA confirmation; L Ladder, 1-8 cDNA amplification with GAPDH.

### 3.2.3 Transcriptional Analysis of TNF-α in Cortex

Real time PCR was carried out to observe the expression of TNF-α in all four groups in cortex *(Figure 3.5A).*When compared with control (1.0 ± 0.0) increased expression ($p<0.01$) of TNF-α was observed in cortex of metals+HFD (1.53 ± 0.08). After Shogaol treatment, the levels of TNF-α was decreased ($p<0.05$) in metals+HFD+Shogaol 2mg (1.04 ± 0.08) group when compared to metals+HFD (1.53 ± 0.08). Significant lowered expression ($p<0.001$) was also seen in metals+HFD+Shogaol 12mg group (0.80 ± 0.04) as compared to metals+HFD group ( 1.53 ± 0.08) *(Figure 3.5A).*

### 3.2.4 Transcriptional Analysis of TNF-α in Hippocampus

In the hippocampus,expression of TNF-α was increased ($p<0.05$) in metals+HFD (1.53 ± 0.13) group as compared to control (1.0 ± 0.0). TNF-α showed decreased expression levels ($p<0.05$) in metals+HFD+Shogaol 2mg group (0.94 ± 0.11) as compared metals+HFD group. While there was significant decreased expression ($p<0.01$) observed in metals+HFD +12mg group (0.79 ± 0.05) when compared to metals+HFD group (*Figure 3.5B*).

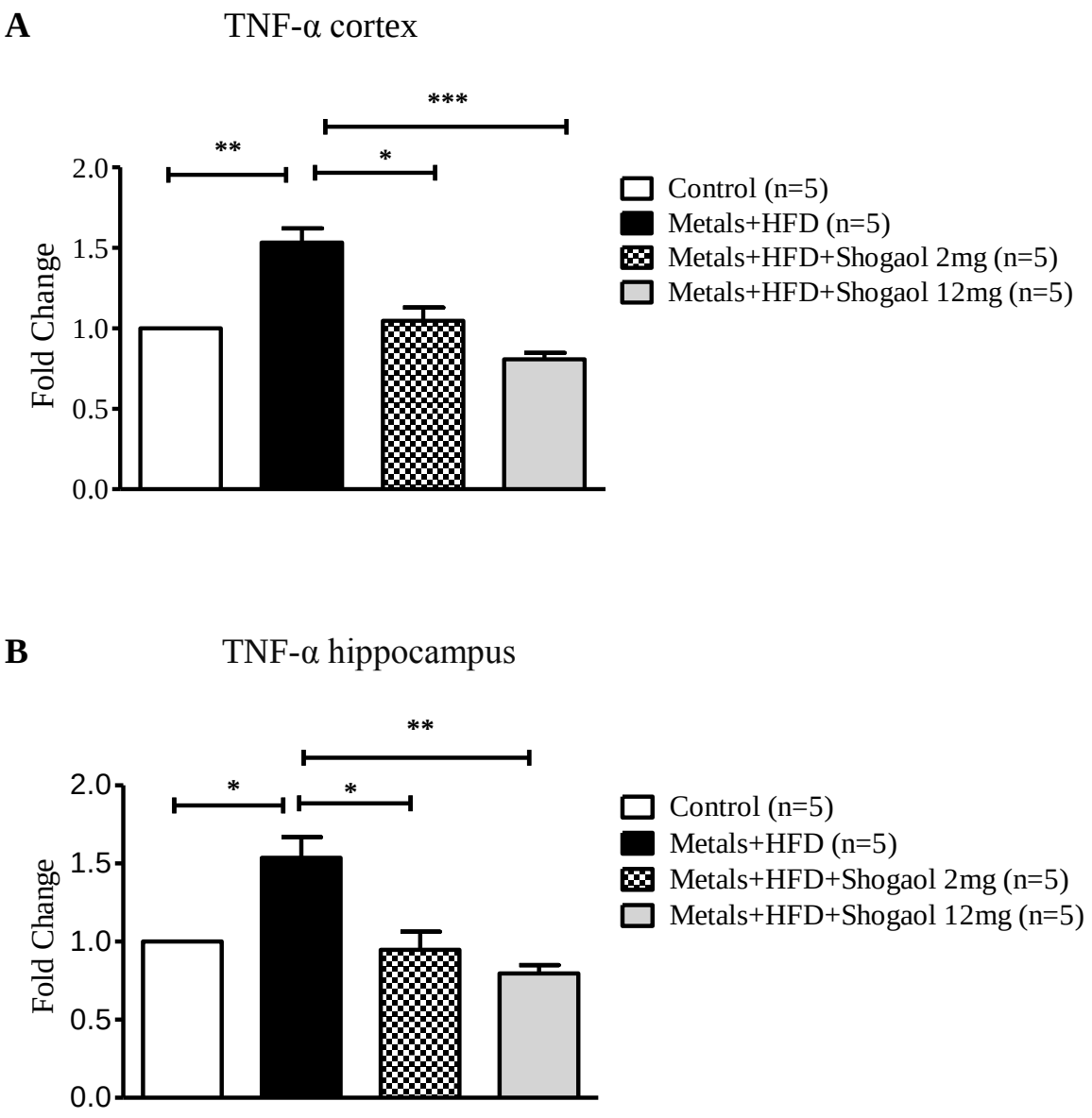



**Figure 3.5:** Histogram representing the relative expression of TNF-α levels in the cortex (A) and hippocampus (B) in control, metals+HFD, metals+HFD+Shogaol 2mg and metals+HFD+Shogaol 12mg group. The data were analyzed using one way ANOVA followed by Bonferroni multiple comparison tests and is shown as mean ±SEM ***p=0.001 **p=0.01, *p=0.01.To check the consistency samples were run in duplicates.

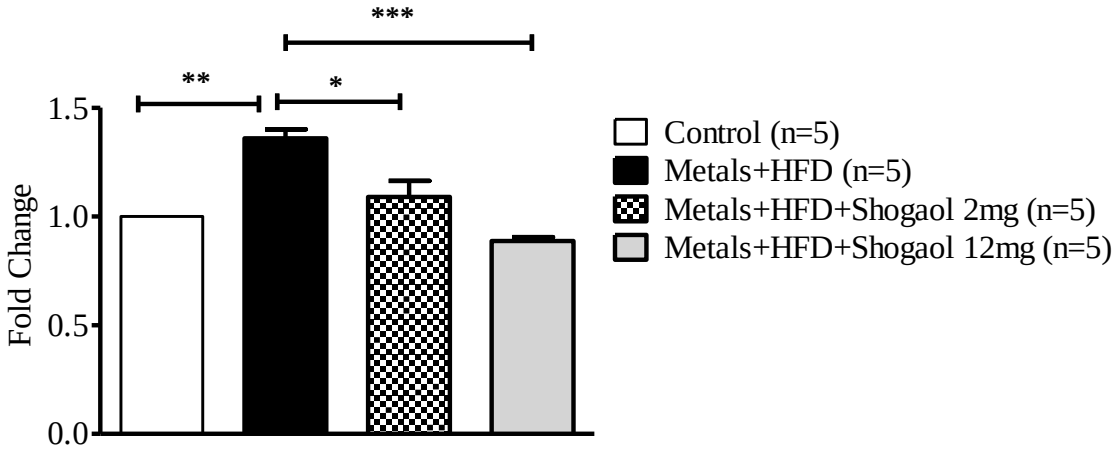
### 3.2.5 Transcriptional Analysis of GFAP in Cortex

There was a substantial increase of GFAP expression levels in cortex *(Figure 3.6A*) ($p<0.01$) of metals+HFD group (1.36 ± 0.04) as compared to control (1.0 ± 0.0). After Shogaol exposure the expression level of GFAP in metals+HFD+Shogaol 2mg group (1.09±0.07) was decreased ($p<0.05$) when compared to metals+HFD group (1.36 ±0.04). The expression of metals+HFD+Shogaol 12mg (0.88 ± 0.01) was significantly decreased ($p<0.001$) as compared to metals+HFD group *(Figure 3.6A*).

### 3.2.6 Transcriptional Analysis of GFAP in Hippocampus

Increased expression ($p<0.05$) of GFAP *(Figure 3.6B*) was observed in metals+HFD group (1.51 ± 0.14) as compared to control (1.0 ± 0.0). There was no significant difference between metals+HFD group and metals+HFD+Shogaol 2mg group but a non-significant trend was observed, metals+HFD+Shogaol 12mg showed significant decreased expression ( $p<0.01$) in comparison with metals+HFD group (*Figure 3.6B).*

**A** GFAP cortex




**B** GFAP hippocampus

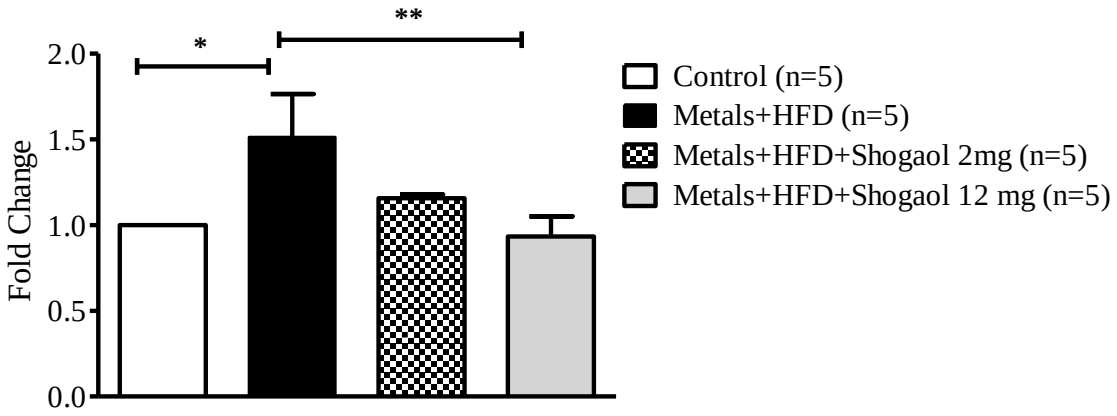



**Figure 3.6: Histogram showing relative expression of GFAP in cortex (panel A) and hippocampus (panel B)**. The data were analyzed using ANOVA followed by Bonferroni multiple comparison test and is shown as mean ± SEM indicates ***p =0.001,*p= 0.01 *p=0.05.Samples were run in duplicates.

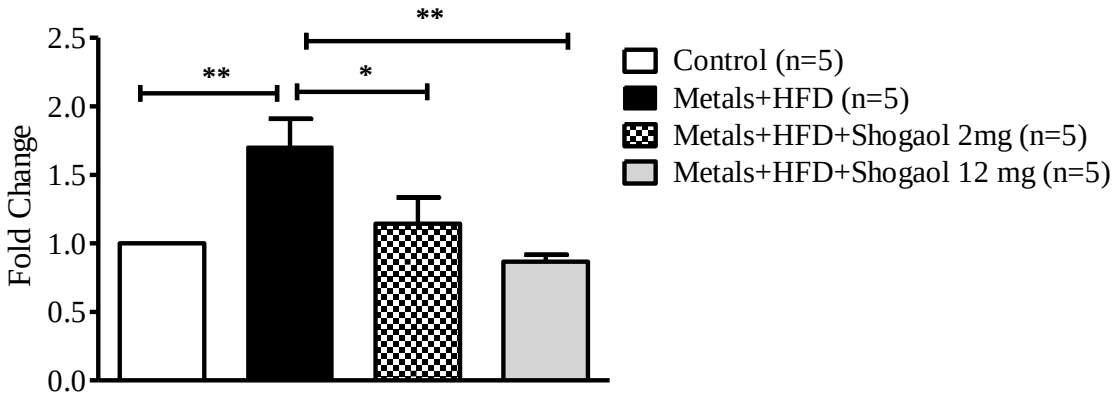
### 3.2.7 Transcriptional Analysis of IL 1 β in Cortex

Real time PCR was done to check the expression level of IL 1 β in cortex (*Figure 3.7A*) which showed that in comparison to control (1.0 ± 0.0) increased expression ($p<0.01$) was observed in metals+HFD group (1.70±0.11), while Shogaol administration caused a decreased expression ($p<0.05$ ) in metals+HFD+Shogaol 2mg (1.143 ± 0.11) group and a significant decreased expression ($p< 0.01$) in metals+HFD+ Shogaol 12 mg (0.866 ± 0.02) group as compared to metals+HFD group (*Figure 3.7A).s*

### 3.2.8 Transcriptional Analysis of IL 1 β in Hippocampus

There was increased expression ($p<0.05$) in hippocampus *(Figure 3.7B)* of metals+HFD group (1.513 ± 0.14) when compared to control (1.0 ± 0.0). IL 1 β expression did not vary among metals+HFD group (1.513 ± 0.14) and metals+HFD+Shogaol 2mg group (1.15 ± 0.012), metals+HFD+Shogaol 12mg (0.93 ± 0.06) group showed increased expression ($p<0.01$) levels of IL 1 β as compared to metals+HFD group *(Figure 3.67B).*

**A** IL 1 β cortex




**B** IL 1 β hippocampus

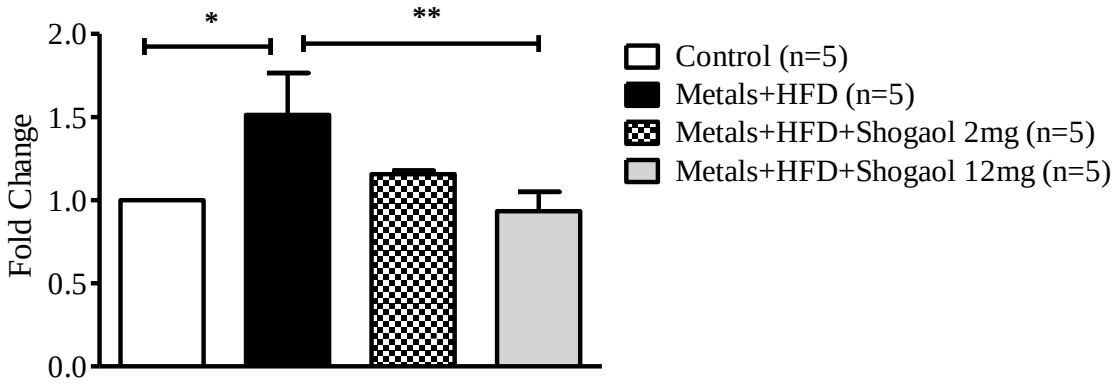



**Figure 3.7:** Graphs showing relative expression of IL-1 β from cortex (A) and hippocampus (B). The data were analyzed using ANOVA followed by Bonferroni multiple comparison test and is shown as mean ± SEM indicates ***p =0.001, **p= 0.01,*p=0.05.Samples were run in duplicates.

***Chapter 4***

# DISCUSSION

In this study we aimed to determine the deleterious effects of heavy metals and high fat diet on certain aspects of neuroinflammation. We furthermore intended to identify and evaluate the effect of Shogaol (natural compound from ginger) on neuroinflammation, BBB disruption along with its effects on inflammatory markers.

Heavy metals affects every organ of the body but CNS is more vulnerable even when administered at small quantity. Contaminated water is the main source of heavy metals exposure. As is widely distributed in the environment and cause serious health problems (Sun et al., 2018). Due to high aluminium exposure, nervous system is compromised and loss of neurons occurs (Cui et al., 2016) which results in cognitive deficits and motor disabilities. It was studied in mice model which clearly demonstrated that metals exposure leads to increased anxiety, BBB disruption, cognitive dysfunction and triggered the activation of microglial cells (Aschner, 2009). The results are in consistent with these studies proposing when mice model are exposed to metals (Al, As, Pb) in drinking water it cause neurotoxicity, alterations in the BBB composition and ultimately leads towards neuroinflammation which effects the normal functioning of neurons.

A study was conducted in animal model of high fat diet which indicated that HFD is the major cause of obesity and effects the normal structure & function of hippocampus. Altered hippocampal structure ultimately leads to reduced decision making and learning & memory impairments (Molteni et al., 2002). Recent work has shown that HFD is associated with

brain inflammation and it induces upregulation of inflammatory mediators like interleukin (IL-1β) and tumor necrosis factor α (TNF- α) (Molteni et al., 2002).

Neuroinflammation is a key feature of various neurodegenerative disorders that includes Parkinson's disease, Alzheimer's disease, and dementia. Moreover, systemic inflammation have a key part in damaging the BBB & microglial cells that are activated and produce an inflammatory response in which different cytokines are released, oxidative stress occurs reactive nitrogen species and oxygen species are generated. All these factors leads to synaptic impairment which results in neuronal death (Greenwood and Winocur, 2005). The present study determines for the first time the effect of HFD and metals mixture (Al, As, Pb) on mice model exposed to high fat diet and metals for a period 8 weeks that induces neuroinflammation in metals+HFD group as compared to control group as evidence by BBB disruption.

Exposure to metal mixture aluminum, arsenic & lead is the main cause of neurotoxicity than single contact of metals. Exposure to heavy metals results in greater nervous damage and cognitive deficits (Sankhla et al., 2017). High fat diet intake induces inflammation in the hypothalamus as a result of reactive glial cells and upregulation of pro-inflammatory cytokines including IL-1, IL-6, IL-16 and various genes like GFAP, Ionizing calcium adapter binding molecule 1 (Iba1). Neuroinflammation induced by HFD activated and altered various pathways like Nrf2 signaling, MAPK and PI3K/AKT pathways. It needs novel approaches which reduces metabolic problems related to neuroinflammation (Liebrich et al., 2007). Our study conveys new findings in account of exposure to metal mixture and HFD which activates various acute & pro-inflammatory cytokines that induces

inflammation in cortex and hippocampus, increased expression of inflammatory cytokines in brain includes IL-1β, GFAP & TNF-α. Upregulation of these genes are observed in metals+HFD group in comparison to control group due to which neuroinflammation occurs in metals+HFD exposed mice model.

Different methods are available for checking BBB leakage. Previous studies exploit the properties of radioactive compounds used in magnetic resonance imaging (MRI) (Floris et al., 2004) to investigate the permeability of the BBB in case of brain injury (Preston and Webster, 2002). But this method required greater manpower, and it is quite expensive. For assessing BBB damage, Evans blue dye is used for assessing BBB dysfunction in animal models of brain injury and it remain the most common procedure. Evans blue dye is injected either intraperitoneal (Morrey et al., 2008) or intravenously (Belayev et al., 2005) for different time period (Mychaskiw et al., 2000). A study was conducted which allowed Evans blue dye to inject for 20 min. (Hellal et al., 2004). Another experiment permitted 1 h & 2 h Evans blue dye circulation period (Kim et al., 2009). BBB assay was performed to determine how metals and high fat diet combine to effect the brain and to investigate the leakage of BBB by using Evans blue dye. Our data demonstrated that Evans blue stain was injected intraperitoneal for 3 h. To test our hypothesis we used, mice model exposed to metals+HFD for a period of 2 months. The amount of Evans blue accumulation was significantly higher in metals+HFD group as compared to control, this was due to BBB disruption and activation of different inflammatory genes (IL-1 β, TNF α & GFAP) in metals+HFD exposed mice.

Shogaol, a natural compound of ginger which is commonly known for its medicinal properties. Shogaol is the major constituent of ginger. It has potential to show anti-

carcinogenic, anti-inflammatory, anti-apoptotic, anti-oxidant and neuroprotective effects (Jolad et al., 2004). Previous study used mice model of LPS (lipopolysaccharide) for induction of systemic inflammation and results in microgliosis (activation of microglial cells), 6-Shogaol which demonstrated its anti-inflammatory effect on glial cells and directly inhibited the microglial cells. Another findings established the neuroprotective role of 6-shogaol in cell culture of ischemia animal model (Ha et al., 2012).

Shogaol is used to treat various inflammatory disorders. Thus, we hypothesized that Shogaol might regulate neuroinflammation by inhibiting microglial activation in brain. When Shogaol 2mg/kg was administered to mice model along with metals+HFD, it was found that Shogaol exhibited anti-inflammatory effect and decreased the gene expression levels of inflammatory markers (TNF-α, IL-1β and GFAP) in cortex & hippocampus as compared to metals+HFD group. Similarly when high dose 12mg/kg of Shogaol was administered, a significant decreased expression of these inflammatory markers was found in the cortex and hippocampus when compared to diseased group (metals+HFD). Consistent with these results, the present study confirmed that Shogaol 12 mg/kg reduce the permeability of the blood brain barrier and decreased the accumulation of Evans blue dye in the right hemisphere and left hemisphere region of mice brain.

High fat diet and heavy metals exposure induced neuroinflammation which was evidently proved by the activation of inflammatory genes (IL-6, TNF-α, IL-12, IL-1β, and COX-2) and different growth factors in brain tissues. Berberine that is an herbal compound, is used as an anti-inflammatory agent, it decreased the expression of TNF-α, IL-6, IL-12, IL-1ß in brain tissues (Hussien et al., 2018). These findings are associated with the current results

but in this study, Shogaol is used as an anti-inflammatory agent to decrease the expression of inflammatory markers in brain tissues.

## CONCLUSION

Chronic exposure of metal mixture aluminium, lead, arsenic along with HFD is associated with neuroinflammation. Heavy metals and HFD persuaded their toxic effect characterized by BBB disruption, production of pro-inflammatory cytokines (IL-1β, TNF-α) and activation of microglial cells (GFAP) in cortex and hippocampus. Shogaol signifying an anti-inflammatory effect by inhibiting glial cells and alleviating neuroinflammation. Shogaol also normalize the expression of IL-1β, TNF-α, GFAP and reduce permeability of the BBB. There is also a need for more investigation of the combine effect of heavy metals and HFD specifically on brain. The essential mechanisms through which heavy metals and HFD persuades neuroinflammation should be studied in additional pathways. Shogaol could be a good target for drug synthesis and it can be used to treat neurodegenerative disorders that is associated with neuroinflammation.

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