Pharmacological Evaluation of Shogaol on Synaptic Impairment in High Fat Diet and Metals Exposed Mice

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DECLARATION

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

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Amna Liaqat

Master of Science in Healthcare Biotechnology

Registration # 00000204425

DEDICATION

All My Effort is dedicated to the

"MY BELOVED FAMILY"

Especially my Father and Mother

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All praises be to **ALLAH Almighty** the most Merciful, the most Gracious and the all-knowing. Due to His mercy and love, all good and great things are possible. Peace and blessings of Allah be upon the last and final Messenger **Muhammad (S.A.W)** who has always been the source of guidance and knowledge to the humanity forever. And blessed me the courage to get higher education and to complete this manuscript.

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At last but not the least, I cannot forget the sincere love, moral support, countless prayers and endless efforts of **My Parents** for supporting me spiritually throughout my life and support me all the time to achieve this goal.

Amna Liaqat

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List of acronyms

AS	Arsenic
Al	Aluminium
Pb	Lead
CNS	Central nervous system
HFD	High Fat Diet
hr	Hour
SYN	Synaptophysin
PSD95	Post synaptic density 95
PCR	Polymerase chain reaction
qPCR	Quantitative real time PCR
PFA	Paraformaldehyde
bp	Base pair
kg	Kilogram
M	Molar
µl	Magnesium Chloride
mL	Microliter
µg	Microgram
AS	Microgram

Abstract

The normal function of the brain is based on millions of neurons found in the central nervous system (CNS). Research in both humans and animals have revealed that two brain areas, the hippocampus and cortex are essential for the encoding and retrieval of episodic memories. Exposure to different metals in the environment and excessive consumption of high-fat diet is responsible for the impairment of learning and memory storage process. Lead, arsenic and aluminum exposure is mostly reported to be involved in this impairment. Moreover, Synaptic proteins play crucial role in the process of learning and memory. Aim of the proposed study is to analyze the effects of Shogaol on memory impairment in High Fat Diet and Metals Exposed Mice. In the current study, we have employed gene expression analysis of synaptic genes (CAMK4, SYN and PSD 95) and histopathology and cell counting in the regions of cortex and hippocampus to evaluate neurodegeneration due to metals and HFD exposure. This study suggested that metals and HFD significantly down regulated the expression of selected synaptic genes while both the doses of Shogaol improved their expression. Neurodegeneration was observed through histopathological examination along with significant decrease in neuronal cell count in hippocampus and cortex, while Shogaol helped to retain the cell number. In conclusion it can be suggested that Shogaol has beneficial pharmacological effects and can be further evaluated.

INTRODUCTION

1.1 Learning and memory

Brain is the central organ which controls the functioning of our body. Brain is comprised of millions and millions of neurons which accomplish the functioning of the brain. These neuronal cells are arranged in a complex but coordinated manner to regulate the functioning of the brain regions as well as respond to the afferent and efferent signals in central nervous system. (Diana et al., 2010). Along with coordinating the functions of other organs, one of the essential feature of brain is to learn and store the knowledge and information based on previous experiences (Knapska et al., 2012). Decades of research is being carried out and it is a well-known fact today that the learning and memory formation aspect of brain is controlled by two brain regions termed as hippocampus and prefrontal cortex (Jin and Maren, 2015).

Learning followed by memory formation is explained in terms of plasticity. Plasticity is the mechanism by which the learning activities and its data is stored for long term usage and for retrieval at later stages of life (Monfils et al., 2005). Through plasticity new connections are built between the neurons. So neuronal plasticity is necessary for both animals and humans to adopt to the changing surroundings. Hence, it is necessary to localize the physical importance of memory formation and neuronal plasticity (Lashley, 1950).

In the field of neurobiology, learning and memory is one of the most astonishing aspect. It involved studies conducing on identifying which regions of brain are involved in memory formation as well as which neuronal aspects are crucial in maintaining this neuronal

connection, which ultimately leads to neuronal plasticity (Thompson, 1986). In the field of neurobiology, the other major area of concern is the neurogenesis. Neurogenesis is the concept of formation of new neuronal cells in the brain. Earlier it is considered that unlike other organs of the body, the cells in the brain do not go on dividing. But with growing time and research, it is considered that adult mammalian brain cells undergo neurogenesis (Altman, 1962). This concept shifted the knowledge of brain physiology and plasticity concept to a new era of research (Gross, 2000).

When an individual encounter a particular signal from the surrounding, then the initial conscious response to that signal is based on the memory that is stored in both the hippocampus and cortex of the brain. Memory formation is consolidated in such a way that information moves from hippocampal regions to the cortical section until it becomes independent of the hippocampus (McGaugh, 2000).

In order to understand the mechanism by which the brain stores and retrieves the information, it is essential to understand the structural anatomy of the brain. Understanding regional anatomy of brain is necessary to get an insight into the process by which the information is encoded, processed, stored and ultimately retrieved (Eichenbaum et al., 2007). Individuals encounter different things in daily life such as interaction with objects or living organisms etc., and these all information is processed by brain. Sensory neurons are present in the body which perceive signal from different regions of the body, which reach the brain and encodes. Initially these signals are responded by the efferent neurons, and later the information is stored for future responses (Davachi, 2006). This flow of information occurs in a specific pathway as shown (figure 1). These interconnected areas of brain are association area (Diana et al., 2007).

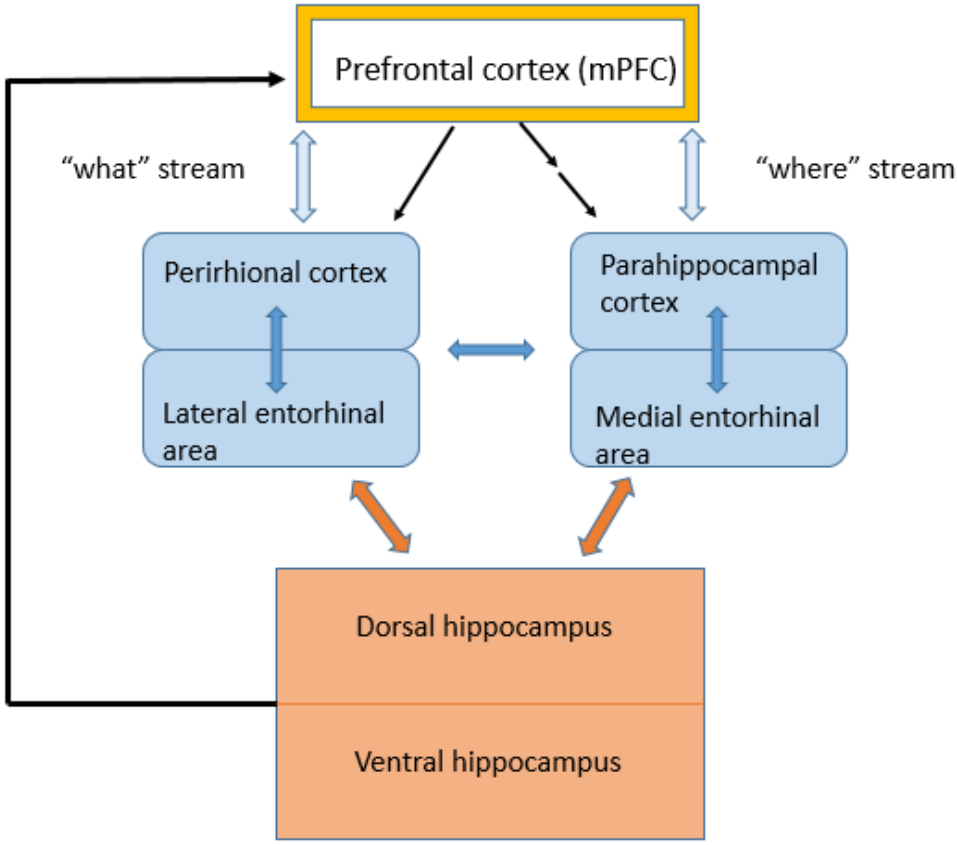

Figure 1.1: Pathways of information flow in between the hippocampus and prefrontal cortex.

Some regions of cortex are dedicated to the function of identifying the spatial memory and to locate where in the space the objects exist. Information related to different types of environment are then collected and transferred to the medial temporal lobe. This MTL is essential for the formation of the event related memory. Both the memories of events related to what and where are then interconnected at the hippocampus level (Diana et al., 2007).Information is processed in the hippocampus which is then transferred back to the cortical regions, i.e., perirhinal and entrorhinal cortex. This interplay of regions assist hippocampus in easy retrieval of information. In this memories are converted into long term memories in the form of strong recollective experiences in human (Fortin et al., 2004). Other than hippocampus and cortex, certain other regions of brain are also involved in the formation of memory and learning processes such as amygdala, but the main focus of this study is only upon hippocampus and cortex and the inter connection of both these brain regions.

1.1.1 Cortex

It is a well-established concept that both hippocampus and cortex interplay with each other in order to process the information and storage of this information in both short term and long term memories as well as for the consolidation of memories (Moscovitch et al., 2016). But it is of vital importance that the cortex and primarily prefrontal cortex is necessary in many of the cognitive functions of the brain and most importantly learning and memory as compared to the other regions of brain. Within the prefrontal cortex, there are distinct areas specialized to receive different type of input from different regions of brains (Bannerman et al., 2001).

1.1.2 Hippocampus

The mammalian brain is composed of two hippocampus in each of the hemisphere of brain which are interconnected to each other and are termed as left hippocampus and right hippocampus. Hippocampus is also crucial in learning and memory formation. But the most important aspect of hippocampus is the neuronal plasticity both in the form of long term potentiation LTP and long term depression LTD (Bannerman et al., 2001). Initially the pioneering data for hippocampus involvement in memory formation was obtained through H.M patient. The study on H.M showed that hippocampus is essential in memory formation along with medial temporal lobe MTL. Removal of these regions from H.M causes symptoms of severe retrograde amnesia. It was also observed that some regions within medial temporal lobe are essential for the formation of declarative memory. Certain regions of hippocampus are involved in different processes of memory consolidation (Corkin, 1984). In the neuronal connections, the primary cells of hippocampus, primary cells are directly activated in response to animal's spatial movement. So as the animal found a specific location in the surroundings, the information is processed in the hippocampus and stored. Also these place cells are involved in processing, forming, retrieval and storage of information related to the events (Naya and Suzuki, 2011).

With respect to long term memory formation, hippocampus is the hallmark in this aspect. It is the main region for memory formation wither long term or short term. Other cognitive functions are also somehow managed in the hippocampus (Hajjar et al., 2013). Hippocampus is itself composed of different regions termed as dendate gyrus, CA1, CA2 and CA3. Movement of signals takes place from dendate gyrus to the CA3 pathway. Each region is involved in consolidating the signals at different levels of storage. CA1 is the first

region which perceives the signals and is responsible for the processes of learning and memory formation. Then CA2 region came into action and is responsible for the converting this memory into long term memory. CA3 cells are required for the neuronal plasticity both LTP as well as LTD (Barton, 1996).

1.2 Synaptic plasticity

During the developmental stages of life, neuronal plasticity is very critical for the development of neuronal circuits and for maturation of neuronal connections. Other than the motor coordination, the visual system of infants is also highly susceptible to the early brain development. Visual system of brain is controlled by the visual cortex (Carew and Sahley, 1986). Different pathways are involved in memory consolidation through different regions of brain, such as CAMK pathway, MAPK pathway etc. one of these mechanisms involves the synaptic strengthening, through neuron transmission. This mechanism is observed both in the gill-withdrawal reflex of Aplysia and in the tail-flick response of crayfish, indicating a decrease in neurotransmitter release is associated with decrease in sensitization as well as decrease in short term habituation (Castellucci et al., 1970). Input to the neurons leads to the sensitization as well as activation of three different types of modulating neurons. These neurotransmitters binds to the receptors at the post synaptic neurons. And causes the influx of ions such as sodium, potassium and calcium. These ions then activate cascade of enzymes in the post synaptic neuron (Bailey and Chen, 1988). Different studies have shown that influx of calcium ions is mainly involved in activating different cascades of proteins, which in turn activates different transcriptional factors, and hence leading to the formation of new neuronal proteins and other proteins involved in plasticity and neurogenesis (Malenka et al., 1989).

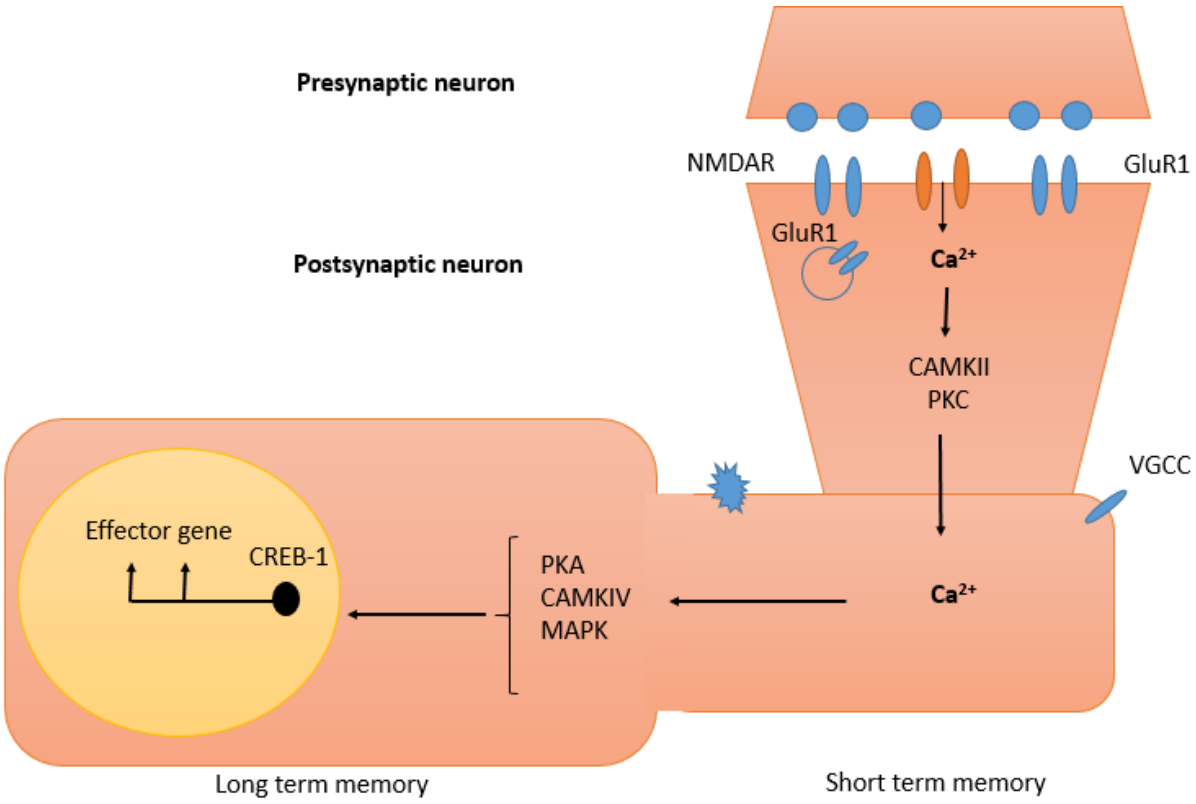

Figure 1.2: Molecular mechanisms for induction of LTP and memory.

1.2.1 Synaptic genes expression profiling

Synaptic proteins are such proteins which are involved in forming the connections between the pre and post synaptic neurons and allows the continuous flow of neurotransmitters and hence successive synapse formation. Such proteins are necessary for a number of functions such as recovery of neurons after any damage, prevention of any neurodegeneration as well as maintaining the synaptic plasticity At structural and functional level, these proteins aid in both the synaptic vesicle formation and signal transduction (Colbran, 2004).Any damage to these proteins will result in alteration at functional at structural level an ultimately cause impairment of memory formation and even synaptic impairment (Wu et al., 2008). Synapse is formed between the axon of one neuron and dendrite of second neuron. Such juxtaposition synaptic neurons forms a synaptic cleft. From the presynaptic neurons, neurotransmitters are released such as serotonin, acetylcholine etc. which enters the synaptic cleft and binds at the receptors present at post synaptic neuron. In this way electrical signals is transmitted to the consecutive connections and action potential is generated. At presynaptic neuron axons are involved and at post synaptic neuron dendritic ends are involved and these structures can be seen by microscopic examination (Hellwig et al., 1994).

The electrical signals all are converged at the same point called synapse. The main purpose of synapses is the timely delivery of signal as well as proper distribution of action potential across the neurons. By altering the mechanism of synaptic signaling, a large number of changes can be brought in the signal transduction leading to alteration in many of the brain function (Mulkey and Malenka, 1992). Synapse can be either inhibitory or excitatory in nature. Both are opposite to each other in function as well as structure. Structurally the

excitatory neuron is composed of dendritic portion covering all the process and isolating the neuron from other surrounding cells (Ngo-Anh et al., 2005).

Connections at synapses are not static but remain in constant form of modifications in response to activity and stimulation by upcoming signals. This is termed as the remodeling of synaptic connections. As a result of this remodeling, there may be different structural changes such as addition of neurons, depletion of neurons or addition of more neurons to a particular synapse at both pre and post synaptic regions. The main force that drives this structural modification is the individual exposure to environment and learning. So these changes are the basis of the process of learning as well as for the formation of memories (Bailey and Kandel, 1993).

Due to toxicity, many regions of brain are affected including hippocampus. In research studies it has been shown that in Alzheimer's cases, there is visible neurodegeneration in the hippocampal regions along with prominent synaptic loss. Neuropathology studies are being conducted and it has been found that hippocampal damage is one of the main and first sign of loss of neuronal cells as well as cognition deficiency (Scheff et al., 2007),so hippocampal damage is the first clue to neuropathy. Hippocampus is the region of brain in which different sub regions are involved in different types of learning and memory processes such as CA1 is involved in learning and memory while CA3 is involved in synaptic plasticity. Pyramidal neuronal structures are present in the CA1 region of hippocampus and are highly affected cells, so they may provide an insight into the diagnosis and suggest an explanation why synaptic loss occurs earlier in Alzheimer's disease. Since synaptic connections are dependent upon different factors such as vesicle formation, its fusion, release of neurotransmitters, binding of NT at receptors and influx of

ions. So it is suggestive that hippocampal damage correlates with the synaptic loses (Blair et al., 2013).So it summarized that synaptic neurons and synaptic proteins are very important in regulating the normal signal transduction and any loss of these connection results in cognitive deficits (Counts et al., 2014)

1.2.2 Role of Synaptophysin in synaptic plasticity

Neurons develop at very early stage as they are needed to coordinate the bodily functions and hence all the differentiation and growth occurs earlier during development. Synapse are essential for the transmission of electrical signals and to develop complex networks. Synaptic functioning is studied using certain markers of synapses (Südhof et al., 1987). Different proteins can be used in this regard including proteins that are involved in production of neurotransmitters or proteins that are involved in vesicle formation. A lot of proteins are used in these function such as synapsin, synaptotagmin and especially synaptophysin. Synaptophysin is the gene involved in formation of synaptic vesicles and maintaining the integrity of these vesicles. It is one of the synaptic protein which was firstly characterized and widely distributed. It is a structural protein and is composed of four trans membrane domains (Sudhof et al., 1987).

1.2.3 Role of PSD 95 in synaptic plasticity

PSD 95 abbreviated as post synaptic density protein is a member of the PSD proteins and is present at the post synaptic neuron. It is one of the most abundant protein of post synaptic neuron. It is a structural protein and acts as a scaffold protein. It maintains the structure of receptors. It is bounded to post synapse membrane (Hsueh et al., 1997). Since this protein is present at the post membrane and maintains the integrity and structure of receptors, so it is believed that this protein is necessary the signal transduction (Kim et al., 1997).

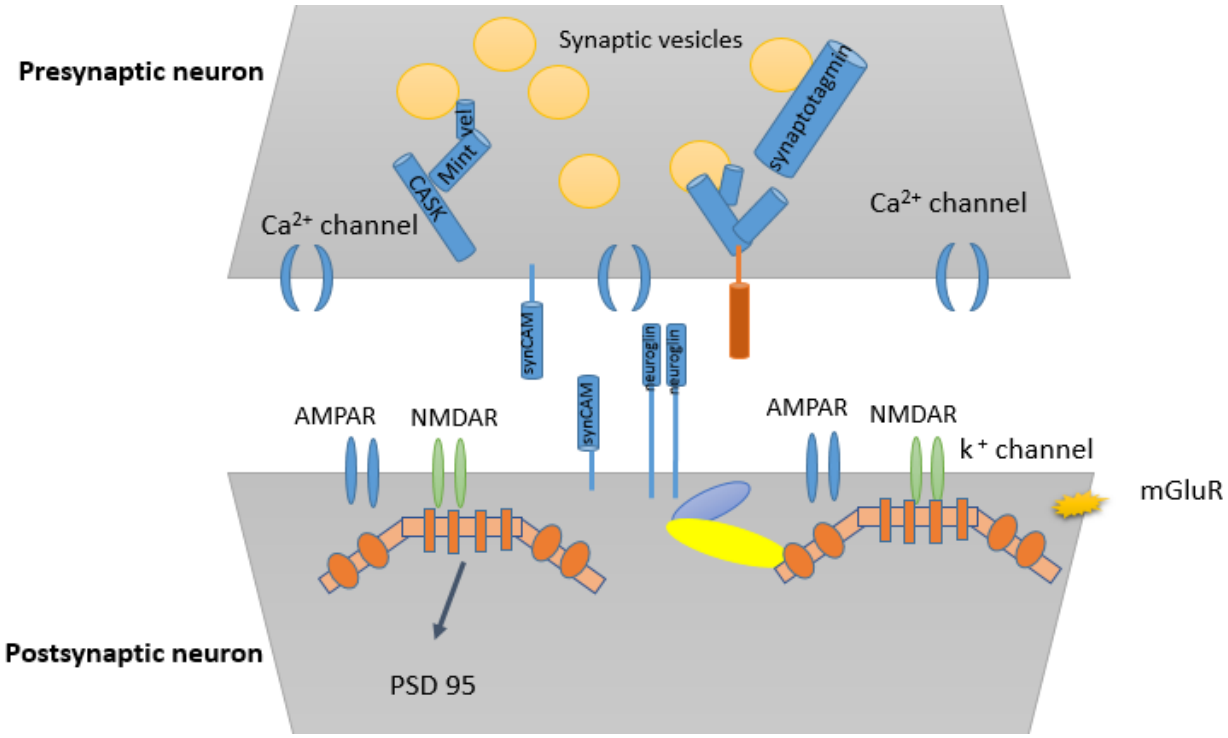

Figure 1.3: Macromolecular PSD-95 complex.

1.2.4 Role of CAMK4 in synaptic plasticity

Certain proteins are essential in the formation of long term memory and synaptic plasticity. For synaptic plasticity and formation of long term memory, a continuous influx of ions and signal transduction is necessary. CAMK4 is one such protein involved in plasticity process through calcium signaling pathway (Silva et al., 1998). Binding of receptors with neurotransmitters causes a rapid influx of calcium ions. These calcium ions then binds to the calmodulin protein within the post synaptic neuron. Binding of calcium of calmodulin protein causes the phosphorylation of different kinase such as CAMK4 and CAMK1 etc. (Malmberg et al., 1997).This CAMK4 is then translated to the nucleus. This is in fact a transcriptional factor for CREB protein. In the nucleus it interact with the CREB and CBP (Ribar et al., 2000). Then it causes the phosphorylation of both these proteins and regulatory factors which then transcribe certain proteins involved in synaptic plasticity, neurogenesis and other essential functions.

1.3 High fat consumption

Nutrients are required by the body. A balanced diet contains specific intake of all type of nutrients and organic material such as proteins, carbohydrates, proteins, fats and vitamins. Any disturbance or abundance of any nutrient can lead to many metabolic disorders as well as cognitive disorders along with hormonal imbalance. Dietary factors can affect the brain functioning in many different ways such as insulin resistance, oxidative stress, inflammation etc., and leading to neuronal damage such as impaired neuronal function or

impaired synaptic plasticity (Gómez-Pinilla, 2008). Among dietary elements, consumption unsaturated fatty acids, folates, vitamins and fish can lead to cognitive decline (Wilcox et al., 2009). High fat containing diet intake is very common in the current era across the world. Consecutive intake of diet with excessive high fat can be a causative factor for the development of certain metabolic diseases such as diabetes, as well as higher cognitive deficits such as induction of dementia, Alzheimer's and neurotoxicity and chronic intake can worsen the situation (Granholm et al., 2008).

Research is being conducted and it has been suggested that chronic intake of diet with high fat content can lead to development and progression of certain metabolic diseases as mentioned previously such as diabetes and obesity. More insight into this aspect and experimentation on animal model has suggested that high fat consumption is also linked to the development of cognitive problems as it causes the damage to blood brain barrier and directly initiate the oxidative stress and hence leading to the neuronal cell death. With the current economic conditions, the intake of high fat diet is increasing worldwide, causing alarming increase in cases of dementia. So need of the hour is to develop new strategies and detailed insight data about how high fat diet intake effects the working and normal physiology of brain (Cordner and Tamashiro, 2015). High fat affects brain and some regions of brain are directly affected such as the hippocampus and cortex. Damage is caused to the hippocampus which in turn impairs the pathways involved in cognitive processes and hence causing cognitive decline (Alzoubi et al., 2018). A lot of literature is available which proposed that high fat consumption has direct impact on cognitive functions as well as it effects the structural morphology of brain. A study conducted by Eskelinen showed the relation of cognitive deficit with fat intake. It was a cross sectional

study conducted on humans to show how fats effect human brain. In this research they observed the intake of fats at midlife and its effect on cognition. They found out that excessive intake of saturated fatty acids leads to cognitive decline (Eskelinen et al., 2008). Many other researcher modified the fat content and studied its effect on brain and especially cognition (Beilharz et al., 2016). Holloway conducted a study in which he studied high fat diet intake and its consequences on brain. He used diet with 75% high fat content and provide it to healthy subjects and undergo observation. Male subjects were only used in this study. They were divided into groups randomly and were allowed to eat either high fat diet with 75% fat content as well as also allowed to eat normal diet with 23% fat content. After a period of 5 days they were tested and it was observed that subjects which took high fat diet were not active and showed impaired attention as well as induction of depression in them (Holloway et al., 2011).

Another study was conducted on high fat diet intake and its effects on cognition by Pistell. He conducted this research on mice models of two types. One group was provided a high fat labelled western diet with fat content of 41% while the other group was provided with high fat lard diet with a fat content of 60%. After development of model, they conducted different behavioral tests as well as in vitro tests. It was found that mice who took 41% fat were prune to obesity with increased body weights as well as astrocytes reactivity, but the cognition was not much impaired. On the other hand high fat diet with fat content of 60% was significant in increasing the body weights of mice as well as induction of cognitive dysfunction as well as inflammation and oxidative stress. Collectively the study suggested that content of fat in diet is necessary in determining its impact on body as well as brain (Pistell et al., 2010). Some other researchers also stated that high fat affects the insulin

signaling pathways in the body, causing insulin resistance, effecting the BDNF gene expression as well as disturbing the inflammatory pathways. It has also been suggested that consumption of high fat diet by mother can cause cognitive abnormalities in off springs as well (Cordner and Tamashiro, 2015).

1.3.1 High fat diet animal model

A lot of clinical evidence is available about the effect of high fat diet consumption on cognition. Animal models are best studied with mice. However, there is still need of more of research based evidence to identify the pathways and genes which are effected by the fat consumption as well as to determine the fat levels which is vulnerable to brain. That is why animal mice models are best suited to conduct such research (Reichelt et al., 2017). Behavioral alterations can be easily studied in animal models. In this way both pathway analysis, behavioral analysis can be conducted. The other positive aspect of animal models is that parallel analysis of brain can reveal detailed information related to morphological alterations in brain such as shrinkage and volume reduction and even cell death can be seen through histological analysis. Even the scientists have demonstrated that chronic exposure of high fat in mice can lead to development of cognitive decline and neurotoxicity by 1 week (Beilharz et al., 2016). However, longer exposure can lead to drastic damage. So it is necessary that timeframe and content of fat is taken into consideration (Stranahan et al., 2008).

A study compared 6-week-old C57BL/6J (6J) mice, using a control group with a low-fat diet (10% fat), and an experimental group consuming a high fat diet (60%), and investigated cognitive and anxiety symptoms. They tested cognitive and behavioral dysfunction with separate groups at 1 week, 3 weeks, or 6 weeks of consumption history,

with each time point having a low-fat control and a high-fat diet group. They found that after 1 week of high-fat consumption, mice had impaired memory as assessed with the Novel Object Recognition (NOR) test. The 3-week consumption history high-fat diet group performed worse in the object learning recognition tasks and the 6-week group showed increased anxiety symptoms in the Open Field Test (OF) and Elevated Zero Maze Task (EZM). This indicates that consumption history differentially affects the outcome of specific tasks, and cognitive and behavioral symptoms vary across consumption history (Gainey et al., 2016).

Dietary animal studies have given us insight into specific brain regions that are more vulnerable to high-fat feeding. These regions include the hippocampus, frontal cortex and thalamus (Cordner and Tamashiro, 2015).

1.4 Metal neurotoxicity

Different environmental risk factors are associated with the onset of brain disorders such as air pollution, high fat diet consumption, smoking, alcohol consumption and metal neurotoxicity. Metal neurotoxicity is one of the most widely distributed factor. Metals are categorized into many levels, some are termed as xenobiotics as they are not needed by the body and their presence even in minor concentration is very toxic for the body. Such metals includes cadmium, aluminum, barium arsenic and mercury etc. these metals at higher concentration are a serious risk to the human body as well as to animals and plants (Carpenter, 1994). The other major problem is that these metals are highly soluble in water which makes their transfer to human body easier. Water pollution is a major concern of Pakistan. Contaminated water is being supplied as drinking water to the population of Pakistan. This contaminated water contains heavy and toxic metals (Waseem et al., 2014)

. The main reason for the consumption of contaminated water is poor economic conditions of the country as well as an imbalance between the economy as well as social development of state. There is rapid shift of population from the rural areas to urban, which causes rapid as well as unplanned expansion. Due to all this, access to clean drinking water is difficult and hence people are exposed freely to the contaminated water (Giller et al., 1998).

Human body is exposed to different variety of metals. Some are useful to body such as calcium while others are harmful such as arsenic. The effect of any certain metal to human depends upon its mobility from the surrounding environment to the human body and then through the pathways it reaches different organs of the body (Jaishankar et al., 2014). A lot of studies are present about the metal pollution and its risk as harmful to human health. With development of any country, industrialization sector grows and metals are widely used elements in industries. Workers at such industries are directly exposed to such toxic metals. In order to resolve this issue, government has provided with a certain range of metal exposure for workers. Since metals are categorized on the basis of safety. Essential metals such as iron, calcium, sodium are required by different organs of body such as brain, blood etc. are necessary for the normal physiology of body (Armendariz et al., 2017). While harmful metals at both acute and chronic levels, are dangerous to different organs of body such as liver, blood and especially brain. Brain is the organ which is highly susceptible to harmful metal exposure. Metals are readily and easily absorbed by the brain. These metals can easily cross the blood brain barrier by undergoing a competitive inhibition. These harmful metals then interact with the normal process of neuronal growth and signal transmission and even disrupts energy homeostasis (Caito and Aschner, 2015).

Certain toxic metals are easily absorbed by the body and get accumulated. These metals have no particular role in homeostasis or any other function. In fact accumulation of these metals even at minor concentration is a health hazard. Some of these metals includes arsenic, aluminum and cadmium etc. The Substance Priority List of Agency for Toxic Substances and Disease Registry (ATSDR) is the institution which categorize the substances based on many factors such as frequency of that element in body, toxicity with respect to availability as well as human exposure. According to this agency, some of the metals such as arsenic, lead, mercury and cadmium are ranked as 1^{st}, 2^{nd}, 3^{rd} and 7^{th}. Not only are these metals present singly in the water, but often human are exposed to a combination of two or more than two metals which can then pose serious risk to humans and animals. When present in combination, these metals show different distribution, availability and effects to the human body (Rodríguez et al., 1998). The toxicity criteria both as toxicokinetics as well as toxicodynamics are modified. This alteration in body response to metals mixture may cause an increased effect as compared to individual effect, a term referred to as synergistic effect. Hence a greater toxic effect is produced. The condition gets much worse when this combined metals target a single organ of the body such as brain. In brain metals mixture attacks different mechanisms such as blood brain barrier causing increased concentration of harmful metals in the body as well as production of free radicals which ultimately decreases the levels of anti-oxidants (Andrade et al., 2017).

Among toxic metals, arsenic is one of the top listed toxic metals. It is a serious hazard to environmental species as well as to humans. Arsenic and its associated chemical compounds are categorized to be carcinogenic (Stueckle et al., 2012). Availability criteria

for arsenic in drinking water is limited by the world health organization at 10 µg/L. not only WHO, but many other private agencies in different countries has also set limits for arsenic in drinking water due to the increasing concern for arsenic as carcinogenic and neurotoxic element. Guidelines are being set by many states for arsenic such as USA and EU. In Pakistan along with other metals, arsenic is also present in higher concentration in drinking water especially in the province of Punjab and Sindh. In Punjab more than 3% water resources are being reported to be containing arsenic at concentration of 50 µg/L, while Sindh has 16% such reported sources. Since WHO criteria is marked at 10 µg/L, so 20% resources in Punjab and 36% resources in Sindh are being reported with arsenic levels of 10 µg/L (Ahmad et al., 2004).

The other toxic metal is lead. Lead is also reported to be highly toxic to human health as well as to plants and animals. Lead has a wide range of effects on human body including metabolic problems to problems related to cognition and even convulsions (Papanikolaou et al., 2005), world health organization has also set the permissible range for lead in drinking water and it is set as 0.001 mg/L. but in Pakistan the levels of lead in drinking water are higher than the WHO range. Collectively the lead levels in Pakistani regions varies from <0.001 to 4.7 mg/L. On individual analysis of different regions of Pakistan, it has been observed that in the samples of drinking water from Azad Jammu and Kashmir, lead concentrations range from 1.8 and 4.7 mg/L (Javaid et al., 2008).

In the south Azad and Jammu Kashmir regions the WHO analysis has revealed that the concentrations of aluminum were about 466 times higher that guidelines. In the KPK state, the lead levels increased due to industrialization and ranges to an average of 0.26 mg/L as compared to critical levels. Likewise, when drinking water samples of Sialkot were

analyzed, it was found that all the samples contained lead concentrations higher than (0.01 mg/L) (Ullah et al., 2009). Also the lead is present in dangerous levels in the atmospheric air of Islamabad. Studies has shown that lead is present in higher concentration across the country but importantly in Islamabad (Agha et al., 2005). Due to recent lead free gasoline, the concentration of lead has decreased, but still the range is not within the safer levels.

Aluminum is another toxic metal. It is abundantly present in the earth crust. It is also used in households and other daily life materials. It is even used in the water purification process and that is why it is extensively found in drinking water (R Walton, 2012). Aluminum is easily taken up the human body due to its easy availability and solubility. It can easily enter and store in different regions of different organs. Brain is the main target of aluminum as it can easily get enters into different regions of brain such as hippocampus and cortex etc. other organs which gets infected by aluminum may include kidney, liver and even bones (Jeffery et al., 1996). But the main target of aluminum remains brain hence this metal is also termed as neurotoxic. In neuropathy, aluminum is considered to be one of the causative agent for Alzheimer's disease. It can also target neural tubes in human brain. A lot of studies are being conducted in this aspect which showed that on exposure to this trivalent cation, organism can easily develop neuropathology, such as shrinkage of brain regions, loss of neuronal cell as well as oxidative stress (de Jesús Ramírez-Altamirano et al., 2012). A study was conducted on aluminum in which rats were provided with intraperitoneal exposure of aluminum for about 90 days along with other supplements of experiment. At the end of experiment it was found that rats developed cognitive impairment involving effects both in motor coordination as well as learning and memory impairment. Rats

showed impaired locomotors activity as well as decline in short and long term memory formation. Enzymes that maintain the levels of oxidants in the brain were also decreased as well as effect on acetylcholine activity (Taïr et al., 2016). Another n vivo study was conducted to check the effect of aluminum on antioxidant enzymes. In this study both developed as well as developing rat brain were analyzed. At the end of study it was found that post aluminum exposure, the levels of antioxidants. Also it was observed that aluminum effects the learning and memory formation process in rats suggesting aluminum is a dangerous neurotoxic (Nehru and Anand, 2005). A study was conducted to check the effect of icariin- a flavonoid on the rats which were earlier exposed to aluminum and to check that either this compound is effective in treating the neurodegeneration induced by aluminum or not. Aluminum was supplied to rats in drinking water for a time lapse of 8 months and then behavior test were conducted post treatment. All the tests indicated that aluminum induced deficits of learning and memory in rats brain (Luo et al., 2007). Exposure to heavy toxic metals can also effect the synaptic signal transmission. A study was conducted to check such effect in which rats were exposed to arsenic metals and expression levels of presynaptic protein was observed. Three presynaptic proteins were tested termed tyrosine hydroxylase, DAT and VMAT2. It was found that DAT was not effected while the other two showed decreased expression (Srivastava et al., 2018). An important point is that the effect of metals on brain depends upon different factors such as the metal concentration, time period for exposure as well as upon the mode of intake of metals. A study was conducted in which arsenic was provided to rats in drinking water, in the form of salt of sodium arsenate. Rats were supplied with metal at different dosage as well as for different time periods. In the end it was found that arsenic exposure induced

learning and memory impairment, deficits of cognitive functions. It also impaired the expression of certain genes involved in maintaining the functions of normal physiology of brain (Sun et al., 2017).

Another study was conducted on investigating the effect of arsenic metal exposure to rats of varying age. In this study the rats were provided with arsenic salt from the gestation day 15 and allowed the intake of arsenic for about 4 months. At the end of the study, certain behavioral tests were conducted to identify any impairment in cognitive functions and memory and learning loss. It was found that rats exposed to metal showed impaired behavior in different tests conducted such as Morris water maze test and also showed locomotors activity deficits as compared to the control group rats which received only distilled water during this period, indicating that arsenic exposure is toxic to individuals and it causes the alteration in brain activity as well as some locomotors activity deficits (Rodrıguez et al., 2002). Similarly another study investigated the arsenic effects on brain and found out that arsenic exposure causes damage at molecular level causing increased expression of dopamine as well as induction of oxidative stress in different regions of brain such as striatum and hippocampus (Yadav et al., 2009). Similar results were obtained by a study conducted on lead exposure (Khodamoradi et al., 2015).

Collectively, a lot of literature is present which showed the individual effect of these metals on nervous system. Few studies are also being conducted on the effect of combination of these metals on brain. Alongside, literature is present which supports the evidence that high fat diet consumption causes an alteration in brain functioning as well as memory loss. There is no clear evidence of using combination of metals and high fat diet and then testing its effect on cognitive functions such as learning and memory.

1.5 Potential of medicinal plants

Plants are a potential source of therapeutic and medicinal compounds. A lot of species of plants are known to possess such active compounds. Currently, approximately, 35,000 species of plant are reported that contain more or less 40.000 active compounds such as phenolic compounds, flavonoids, terpenes and phytochemicals. These compounds are of great health benefits. These natural compounds possesses some therapeutic properties such as anti-inflammatory, anti-diabetic, anti-cancerous, anti-oxidant as well as neuro-protective (Malviya et al., 2010). These properties are of effective use and a lot of research is being conducted on these plant extracts. However plant extract is difficult to be used as pharmaceutical drug as the bioavailability of natural compounds vary, so compound are extracted and can be used for medicinal purpose as both curative as well as preventive medicine (Anekonda and Reddy, 2005).

1.6 Ginger

Ginger is the widely used plant. Its rhizomes are being used in cooking. Scientific name for ginger is *Zingiber officinale*. It is usually added to the food to add flavor and taste of cooking material. However, it is also a very useful plant at therapeutic aspect. Traditionally it was being used for treatment of different ailments. It is directly added to cooking and is also taken as tea. Little is known about the exact mechanism by this plant provides positive effects. It is reported to be providing positive health effects as anti-cancerous, anti-diabetic and anti-oxidant (Grzanna et al., 2005). Now it has been suggested that it may provide neuro-protective effect. Different active compounds are of great health importance. It contain certain compounds such as Shogaol, Gingerol, Zingerbone etc. each compound has

its specific role in wide range of disease prevention. Active compounds of ginger such as gingerol is shown to be providing anti-cancerous effects. Shogaol is shown to be neuro-protective while certain other compounds has shown to be anti-oxidative. It also shows reduction to lipid oxidation (Krishnakantha and Lokesh, 1993).

Focusing on the neuroprotective effect of ginger, it has strong evidence as an effective neuroprotective agent (Shanmugam et al., 2011). The exact mechanism by which ginger shows this useful biological role is still unknown, but it is suggestive that especially flavonoids and phenolic compounds of ginger are involve in this aspect. Among flavonoids and phenols, the majorly involved compounds are gingerol, paradol and importantly Shogaol (Dugasani et al., 2010). Different in vivo studies are being conducted on Shogaol and gingerol showing its neuroprotective effects in mice and rats and being validated through behavior testing as well as molecular analysis (Sharma and Singh, 2011).

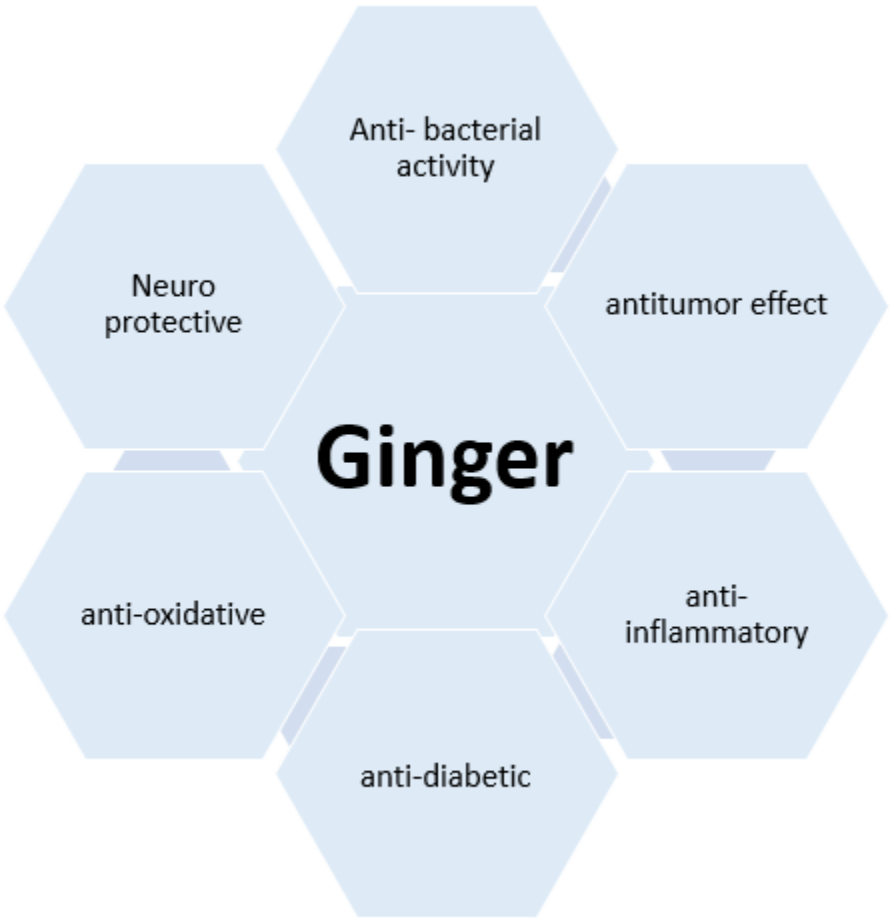

Figure 1.4: Ginger and its constituent's shows role in diseases prevention.

1.6.1 Shogaol

One of the essential compound of ginger is Shogaol. Shogaol like other compounds has shown its potential to treat many metabolic disorders. Shogaol can be obtained from dried ginger.it has a very pungent smell with dark yellowish brown appearance. It is obtained from its precursor gingerol.

Shogaol is a derivatives of Gingerols. 6-Shogaol (6-SG) has been studied to have various biological effects, including anti-inflammatory (Han et al., 2017), anti-diabetic , anti-tumor (Ray et al., 2015), anti-oxidative (Na et al., 2016b), neuroprotective (Na et al., 2016a), anti-amyloidogenic activities (Na et al., 2017). Shogaol is well characterized compounds and its chemical properties are enlisted in Table 1.1.

Shogaol is derived from gingerol. Ginger contain a ketone functional group i.e., beta hydroxyl group and upon heating or providing energy, the ketone group gets saturated and converts the chemical and biological properties of Gingerol and converting it into Shogaol. It has been reported that Shogaol is more effective and potent compound as compared to its parent compound. Like Gingerol, Shogaol has strong effects as anti-inflammatory to neuroprotective effects (Kou et al., 2018). Many Shogaol compounds are formed by thermal dehydration of Gingerol compounds, with 6-shogaol being the most abundant. 6-Shogaol is a promising compound with a number of pharmacological activities, including antioxidant, anti-inflammatory, anti-neuroinflammatory, anti-cathartic, anti-neoplastic and hypotensive effects. 6-shogaol also shows capacities to resist neuronal apoptosis and to modulate synaptic and cholinergic functions. These neuropharmacological activities and mechanisms were summarized in a recent review (Kou et al., 2018).

Table 1.1: Chemical properties of Shogaol compound. Table is adapted from Pubchem Database.

Property Name	Property Value
Molecular Weight	276.4 g/mol
XLogP3	3.7
Hydrogen Bond Donor Count	1
Hydrogen Bond Acceptor Count	3
Rotatable Bond Count	9
Exact Mass	276.172545 g/mol
Monoisotopic Mass	276.172545 g/mol
Topological Polar Surface Area	46.5 A^2
Heavy Atom Count	20
Formal Charge	0
Complexity	299
Isotope Atom Count	0
Defined Atom Stereocenter Count	0
Undefined Atom Stereocenter Count	0
Defined Bond Stereocenter Count	1
Undefined Bond Stereocenter Count	0
Covalently-Bonded Unit Count	1
Compound Is Canonicalized	Yes

A number of studies documented the neuroprotective activity of 6-shogaol in vitro and in vivo. Kim and Kim (2004) reported the neuroprotective effect of ten synthesized Shogaol compounds, including 6-shogaol, in the protection of the rat pheochromocytoma (PC-12) and human neuroblastoma (IMR-32) cells from the β-amyloid insult (Kim and Kim, 2004). It was reported that 6-shogaol protected cholinergic neurons from reactive oxygen species (ROS) in hippocampal neuronal (HT22) cells (Shim and Kwon, 2012).

Ginger has been very well known to be a memory-enhancing agent. However, there has been no evidence of which ingredients of ginger exhibit memory-enhancing effects.in a study, it was found that 6-shogaol administration significantly enhanced learning and memory in both memory-impaired and normal mice (Moon et al., 2014). Therefore, this is the first study to show the effect of 6-shogaol on cognitive function. Data suggests that 6-shogaol seems to directly contribute to ginger-mediated enhancement of cognitive function. The data suggests that 6-shogaol seems to, in part, contribute to ginger-mediated enhancement of cognitive function (Moon et al., 2014).

MATERIALS AND METHODS

2.1 Ethics statement

All the experimentation was carried out in compliance with the rules and guidelines of institute of laboratory animal research, Division on Earth sciences, National institute of health, USA. Each and every protocol used in this work was under the approval of internal review board (IRB), Atta ur Rehman School of Applied Biosciences, national University of Science and technology.

2.2 Chemicals and reagents

Shagoal compound was a generous gift by Dr. Zaman from Allama Iqbal University. Buffalo fat used in construction of animal model was purchased and processed. Reverse trascriptase, deoxyribonucleotide triphosphate, taq polymerase were obtained from ThermoScientific.

2.3 Animals

2-3 weeks old Male Balb/c mice were used in this study. They were obtained from national institute of health, NIH Islamabad, Pakistan as well as from ASAB animal house facility. Animals were kept at the laboratory animal house of ASAB, NUST. Animals were acclimatized to the conditions of animal house for two weeks. Before starting the experiment, animals were kept in cages. A steady temperature of 25±2 and a regular light-dark cycle (12L-12D) was maintained (Iqbal et al., 2016). Regular feed and distilled water was provided prior to experiment to mark the average food and water intake. A total of 48 mice were used in this experiment.

2.4 Construction of mice model

In this study, preventive treatment model was used. Neurotoxicity and impairment in brain was induced by using high fat diet and metal mix. High fat diet was prepared by mixing normal feed and buffalo fat at the ratio of 60:40. Fat was heated and then mixed with the normal feed. For treatment purpose, feed was mixed with Shagaol compound too. The protocol used for this purpose was to make the dilution of Shagaol in 1ml ethanol and then respective volume of Shagaol was added to the high fat i.e., 2m/kg for low dose feed preparation and 12mg/kg for high dose feed preparation. Feed prepared was allowed to air dry overnight and then packed in zip lock bags. Air drying removes the ethanol content as well. Aluminum, lead and arsenic were provided mixed in the distilled water. Before starting the model development, average feed and water intake of mice was observed.

2.5 Study design

Study comprised of 60 days trail. Mice were divided into four groups. Each group consisted of 10 male mice. Group 1 comprises of the control mice. These mice were provided with the normal feed and distilled water for 60 days. Group 2 labelled as the diseased was provided with high fat diet as well as metal mix in dH_2O. group 3 labelled as low dose treatment group received feed containing high fat as well as Shagaol compound along with metal mix in dH_2O and group 4 labelled as high dose treatment group received feed containing high fat as well as 12mg/kg of Shagaol along with metal mix in dH_2O. At the end of preventive model phase, the mice were dissected and RNA was extracted for gene expression analysis and as well as perfused to get histological examination done.

Table 2.1: Table shows the division of mice in groups

Group No.	Description	No. of Mice
1	Control	12
2	Metals+HFD	12
3	Metals+HFD+Shogaol(2mg)	12
4	Metals+HFD+Shogaol(12mg)	12
Total No. of mice		48

2.6 Brain dissection and isolation of cortex and hippocampus

Chloroform was used to anesthetize the mouse. Target organ is brain so the head of mouse was stretched forward, in order to make a cut at the posterior side of ear using the surgical scissors. First a small cut was made from the caudal point of the brain, followed by sharp cuts at the posterior side of skull. Then curved forceps were used slid under the anterior part of the brain in order to lift up the brain. The brain was then transferred to the petri plate which was kept chilled by ice and covered with aluminum foil. 70% ethanol was sprayed prior to placement of brain in order to prevent any degradation.

Firstly the olfactory bulb and cerebellum of the brain were removed. Using small forceps, the cortical halves were opened, by holding the forceps at closed position between the cerebral halves. Samples of cortex were collected and from the opening, the hippocampus was also collected and transferred to the eppendorf and either stored at -80◦C for further use or directly 1ml trizol is added for RNA extraction purpose.

2.7 Gene expression analysis

2.7.1 RNA extraction

RNA extraction was performed according to the manufacturer's protocol utilizing tri-reagent. After dissection and collection of hippocampus and cortex samples, 1ml of trizol was added. The mixture was then homogenized utilizing a sonicator (UP400S heilscher Ultrasound technology). The homogenized solution was allowed to incubate for 10 minutes at 4◦C to ensure the complete homogenization and dissociation of nucleoprotien complex. After that, 200µl of chloroform is added and shaken well. Shaking ensured that mixture turned milky and again incubated for 5 min at 4◦C. the tubes were then allowed to

centrifuge at conditions of temperature 4◦C for 20 minutes at the speed of 12000 rpm. After completion of centrifugation, the upper transparent layer was collected in separate tubes and 500µl of isopropanol was added to it. It followed an incubation for 10 min at 4◦C. again the tubes were centrifuged for 20 min at 4◦C with speed 12000 rpm. It resulted in pellet formation at the bottom of the tube. Supernatant was discarded and 1ml of chilled 80% ethanol was added. It again followed centrifugation at 4◦C for 5 min at the speed of 7500 rpm. Then ethanol was discarded and 30µl of DEPC water was added to the pellet and stored at -80◦C until further used.

2.7.2 Quality of RNA

Prior to the preparation of complementary DNA, RNA was accessed both qualitatively and quantitatively. RNA was quantified using the Thermo Scientific NanoDrop, while the quality of RNA was determined by running it on agarose gel. 1% agarose gel was used for RNA prepared in TBE buffer. Gel was run at 400mA and 90 V for 40 minutes. Later the gel was visualized using wealtechDolphen Doc (S/N 4708830, Wealtec biosciences Co., USA). 18S and 28S bands of ribosomal RNA are ensured and compared among all the samples.

2.7.3 cDNA synthesis

For the synthesis of cDNA, RNA was taken as per concentrations determined through nanodrop. cDNA synthesis was carried out according to the protocol of Thermo Scientific. 10mM oligodT, nuclease free water and RNA sample were incubated at 65◦C for 5 min. then remaining chemicals were added as 10mM dNTP's , 5X reverse transcriptase buffer (Thermo Scientific) and reverse transcriptase enzyme (Thermo Scientific) (Table 2.2). The thermo cycler profile used for cDNA synthesis is shown (Figure 2.1).

Table 2.2: chemical recipe used for the synthesis of cDNA

Component	**Quantity**
RNA	3µg
OligO DT Primer	1µl
Nuclease free water	Up to 13µl
5X RT buffer	4µl
10MM dNTP'S	2µl
Revert AID	1µl

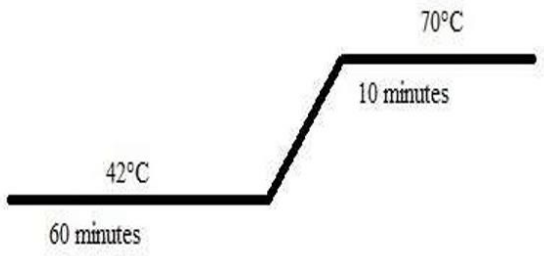

Figure 2.1: Thermocycling profile used for cDNA synthesis

2.7.4 Primer designing

Primers for real time PCR were taken from the literature (Table 2.3) and were confirmed for homology using insilico software, UCSC genome browser InSilico PCR.

2.7.5 cDNA confirmation through conventional PCR

In order to confirm the synthesis of cDNA, conventional PCR reaction was run. Housekeeping gene GAPDH was used in the reaction. For cDNA synthesis protocol from Thermo Scientific was followed. Total reaction volume was set at 25µl. constituents of the reaction mix are mentioned in the (Table 2.4) along with the thermo cyclic profile (Figure 2.2).

Table 2.3: Real time primers and their annealing temperature

Gene	Primer sequence 5’ to 3’	Annealing temp.	Reference
GAPDH F primer GAPDH R primer	CCTGGCCAAGGTCATCCAT GTCATGAGCCCTTCCACGAT	60◦C	From LAB
CAMK4 F primer CAMK4 R primer	CGAAGATGCTCAAAGTCACGG ACTCCACCTCGAAGAAATCGC	60◦C	(Ahmed et al., 2010)
Synaptophysin F primer Synaptophysin R primer	CATTCAGGCTGCACCAAGTG TGGTAGTGCCCCCTTTAACG	60◦C	(Shirendeb et al., 2011)
PSD F primer PSD R primer	′ GGACATTCAGGCGCACAAG TCCCGTAGAGGTGGCTGTTG	60◦C	(Shirendeb et al., 2011)

Table 2.4: chemical recipe used for the confirmation of cDNA

Constituent	**quantity**
cDNA	2 µl
10X reaction buffer	2.5 µl
10mM dNTP	1 µl
25mM $MgCl_2$	2.5 µl
Forward GAPDH primer	1 µl
Reverse GAPDH primer	1 µl
DNA polymerase	0.5 µl
water	14.5µl

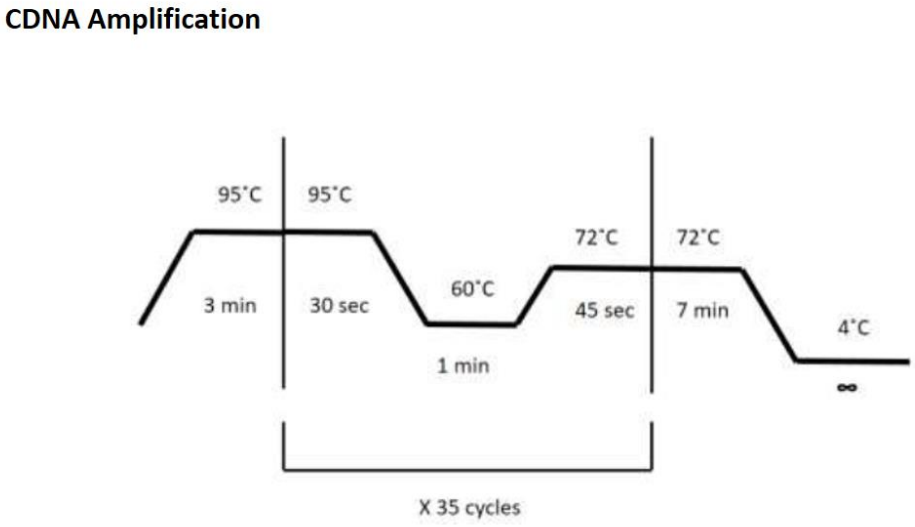

Figure 2.2: Thermocycling profile used for cDNA confirmation by conventional PCR

2.7.6 Gene expression analysis by quantitative real time PCR

ABI prism 7300 sequence detection system (Applied Biosystem, 7300) was employed for real time PCR. Master Mix of 20µl was prepared. Master Mix consisted of maxima SYBR Green/ROX, forward and reverse primers of target gene Constituents of the reaction are shown (Table 2.5). The thermo cycling conditions are shown (Figure 2.3).

2.7.8 ΔCT calculation

All the reactions were normalized to the house keeping gene, so ΔCT was calculated to normalize the target genes. Following formula was used.

$\Delta CT = CT_{\text{target gene}} - CT_{\text{housekeeping gene}}$

2.7.9 ΔΔCT calculation

ΔΔCT value was calculated using the following formula.

$\Delta\Delta CT = \Delta CT_{\text{target group}} - \Delta CT_{\text{control}}$

2.7.10 Fold change

In order to determine the fold change $2^{-\Delta\Delta CT}$ was determined.

2.7.11: Statistical analysis

Statistical analysis of the real time PCR results was performed using GraphPad Prism and Microsoft excel analysis. One way ANOVA was performed to identify the relation between variables. Graphs were designed using the GraphPad Prism version 7.

2.7.7 Expression analysis

Expression of target genes was analyzed using livak method ΔΔCT (Livak and Schmittgen, 2001).

Table 2.5: chemical recipe used for the real time PCR

Constituents	quantity
2X maxima SYBR green/ ROX	4 µl
Forward primer	1 µl
Reverse primer	1 µl
PCR water	13 µl

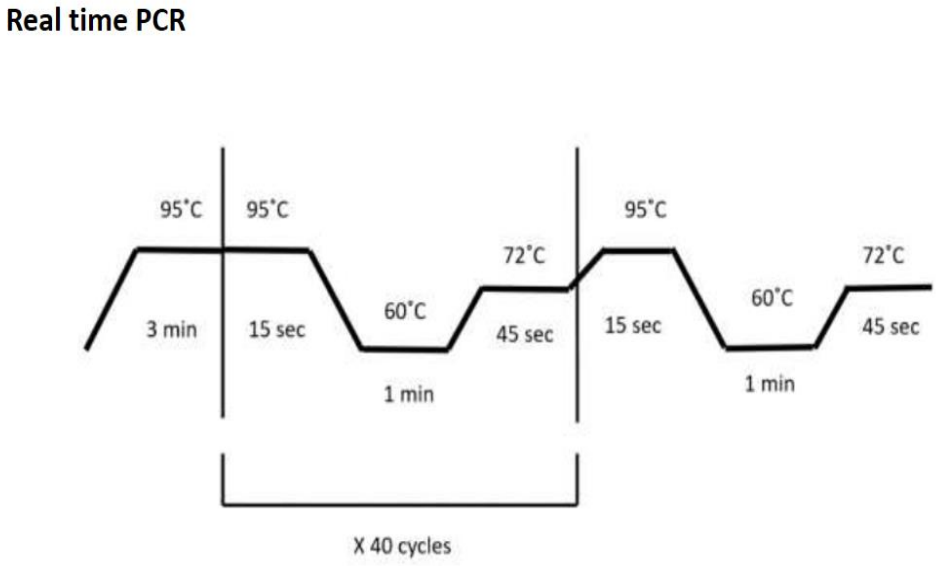

Figure 2.3: Thermocycling profile used for real time PCR

2.8 Histological examination of brain regional tissues

2.8.1 Tissue perfusion

Heart perfusion was carried out according to the protocol by (Gage et al., 2012) . Mouse was anesthetized and cut open in order to get access to the heart. Butter fly needle of 23 guage was inserted into the left ventricle of heart. It was inserted at the apex of ventricle. 70-95ml of 0.9% saline solution was allowed to pass through the heart. In the meantime a cut was made at the right atrium. It allowed the saline solution to pass through the organs of mouse. Later, 4% freshly prepared paraformaldehyde was allowed to pass through the organs. After the completion of heart perfusion, mouse brain was excised and was placed in 4% paraformaldehyde at 4◦ C for 24 hours.

2.8.2 Dehydration and fixation for histological assessment

After 24 hours in paraformaldehyde, brain was passed through the process of dehydration with different dilutions of ethanol. 70%, 95% and 100% ethanol was used in duplicates. Brain was shifted into each dilution for 1 hour. Then brain was placed in xylene-dehydrating agent for 4 hours. In the end brain was shifted to preheated paraffin at 60◦C for 4 hours. After 4 hours block was formed and kept at 4◦C until next used.

2.8.3 Adherent slide preparation

Adherent slide were prepared according to the protocol provided by (devBio.net). Equal proportion of glycerin and egg white were added and mixed for 30 minutes. The solution was then preserved at 4◦C and can be used for up to 1 month.

Tiny drop of the adherent mixture is placed on the slide and thin layer is gently applied using tissue paper. The slides are then placed at 37◦C to ensure adherence.

2.8.4 Tissue sectioning through microtome

Rotary microtome CUT 6062 was used for tissue sectioning. Sections of 6µm thickness were cut. Both the block as well as blade were kept cold in order to prevent the blocking. Sections were first placed in cold water for few seconds and then into the cold water preheated to 40◦C. sections were then settled on the glass slide, and placed at 70◦C to ensure proper adherence of brain section to slide.

2.8.5 Cresyl violet staining

Cresyl violet stain preparation and staining process was performed according to the protocol provided by neurosciences.com. Sections of 5µm mounted on the slides were placed at 70◦C for a few minutes in order to ensure the adherence. Then staining was carried out in dark as mentioned in (Table 2.6). After completion of step 10, slides were covered with the cover slip using the DPX mounting media and placed at an angle of 45◦ and allowed to dry.

2.8.6: Image visualization

Slides were observed under the light microscope, and images were taken using the software Optika Vision Lite 2.1.Images were taken at different resolutions including 4X, 10X and 40X.

Table 2.6: Cresyl violet staining steps

Steps	chemical	Time (min)
1st step	xylene	5
2nd step	95% alcohol	3
3rd step	70% alcohol	3
4th step	dH_2O	3
5th step	stain	8-14
6th step	dH_2O	3
7th step	70% alcohol	3
8th step	95% alcohol	1-2
9th step	100% ethanol	Up to 10 dips
10th step	Xylene	5

2.9 Quantification of cell number

2.9.1 Quantification of cell number in cortex

Quantitative analysis of the cell number was carried out in cortical layers (Layer 1, Layer 2-3, Layer 4, Layer 5 and Layer 6) in the areas of motor cortex and somatosensory cortex (hind limb and fore limb region). The analysis was performed in area of 7500 μm^2 (Figure 2.4 A-B) manually from four randomly selected regions in each cortical layer.

2.9.2 Quantification of cell number in hippocampus

Similarly cell number was quantified in hippocampus from dentate gyrus (DG), CA1, CA2 and CA3 regions. From each region three areas of 245000 μm^2 were randomly selected and cell number was quantified in these areas (Figure 2.5 A-B)). Later the average of values from all four areas in each layer of cortex and all four regions of hippocampus were taken and were plotted.

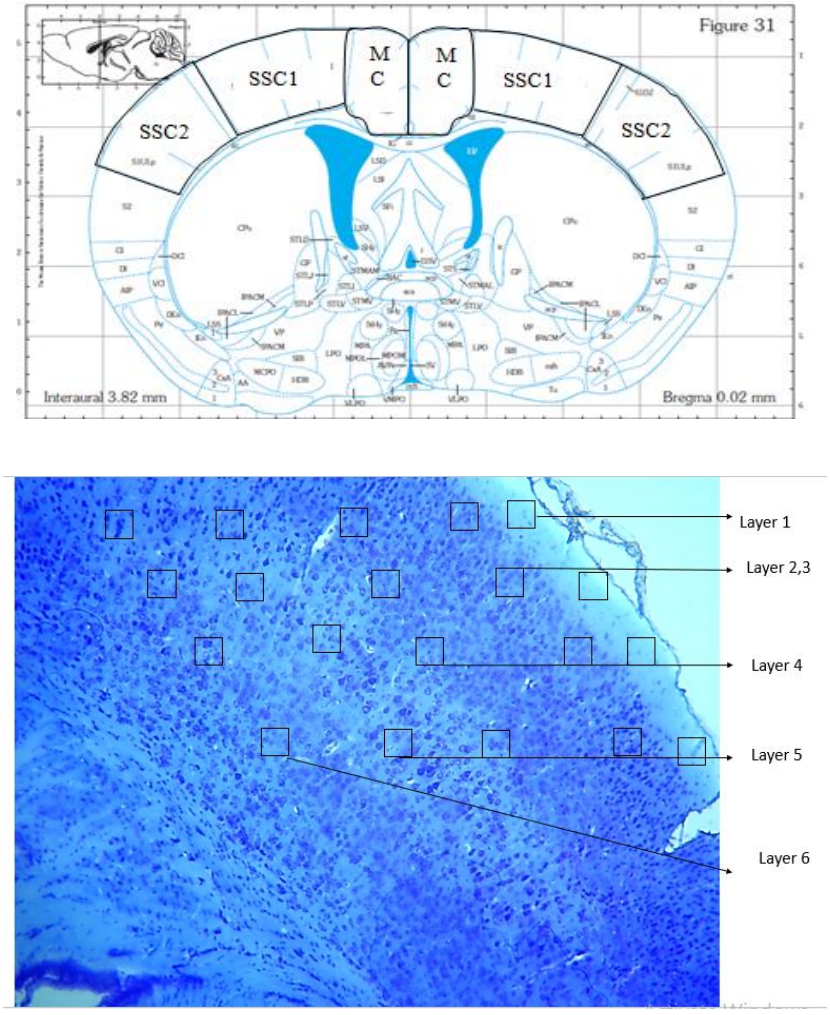

Fig 2.4: Quantification of cell number in cortex: (A)Presentation of the mouse brain atlas coordinates from where the sections were taken for histological examination and quantification of cell number at magnification 10X. MC: motor cortex, SSC1: somatosensory cortex (B) representative slides showing regions of cell count.

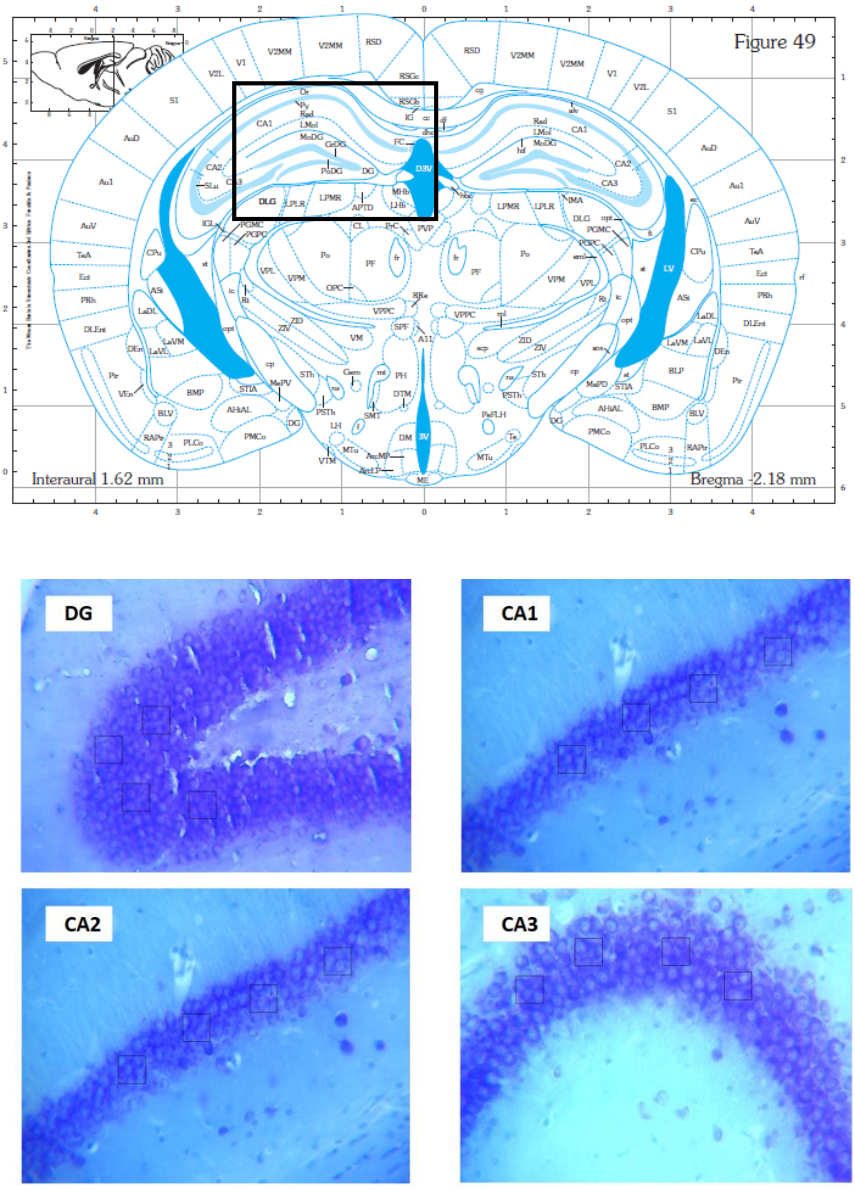

Fig 2.5: Quantification of cell number in hippocampus: (A) Presentation of the mouse brain atlas coordinates from where the sections were taken for histological examination and quantification of cell number at magnification 40X. MC: motor cortex, SSC1: somatosensory cortex (B) representative slides showing regions of cell count.

RESULTS

In order to evaluate the pharmacological effects of shogaol in learning and memory impaired mice models, expression profiling was performed. Expression of gene related to the synaptic plasticity i.e., CAMK4, synaptophysin and PSD95 was accessed through qPCR. Changes in hippocampus and cortex at tissue level were examined through histopathological examination. Neuronal cell death was examined through cell counting.

3.1 Expression studies by qPCR

3.1.1 RNA extraction

RNA extraction was carried out by TRIZOL method and was confirmed qualitatively through gel electrophoresis. The two bands showed the subunits of ribosomal RNA (Figure 3.1). The RNA quantity was measured by Nano drop.

3.1.2 cDNA amplification

cDNA synthesis was confirmed by amplification of housekeeping gene GAPDH through conventional PCR (Figure 3.2).

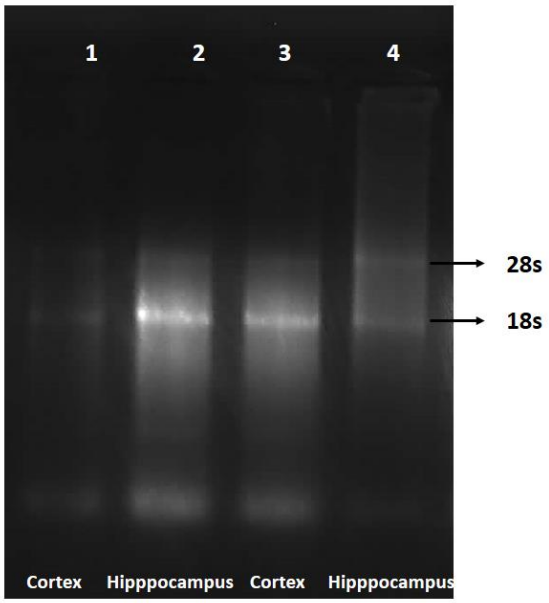

Figure 3.1: Gel containing bands of RNA in Lane 1 to 4, subunits of ribosomal RNA: 28S and 18S are shown.

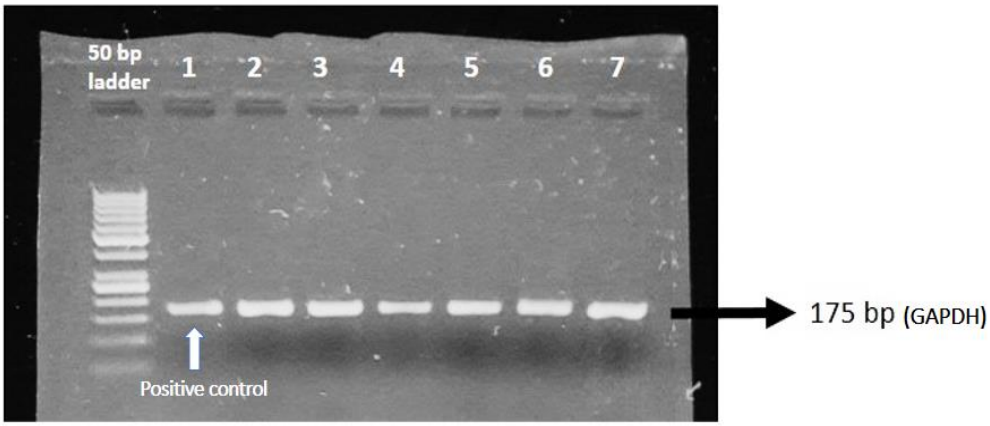

Figure 3.2: cDNA confirmation through conventional PCR: The figure shows the cDNA amplification product using GAPDH gene. Left most lane contain 50bp ladder. Lane 1 to 7 contain cDNA product. Lane 1 is positive control and lane 2-7 contain GAPDH product at 175bp. Lane 1 has positive control while other lanes have GAPDH (housekeeping gene) having 175 bp size.

3.1.3: Primer optimization

Primers used in this study were optimized through gradient PCR in order to identify the precise annealing temperature. All the three synaptic genes showed their annealing at 60◦C. Gradient PCR product was run on 2% agarose gel. 50bp ladder was used to confirm the product size as shown (Figure 3.3).

3.1.4 Primer efficiency

Primer efficiency was calculated for all the genes Synaptophysin, PSD95 and CAMK4 genes by making serial dilutions of cDNA. cDNA was serially diluted and then after the completion of PCR run the ct values were plotted on y-axis and log of cDNA dilutions was plotted on X-axis. A trend line was drawn and linear equation was represented. From the equation, the value of slop was obtained and then put into the formula [E = -1+10(-1/slope)] for calculating primer efficiency. The primer efficiency of Synpatophysin gene was 106.28% and graph is shown in (Figure 3.4). Primer efficiency of PSD95 was 104.44% and graph is shown in (Figure 3.5). For CAMK4 gene, primer efficiency came out to be 99.79% and the graph is represented in (Figure 3.6).

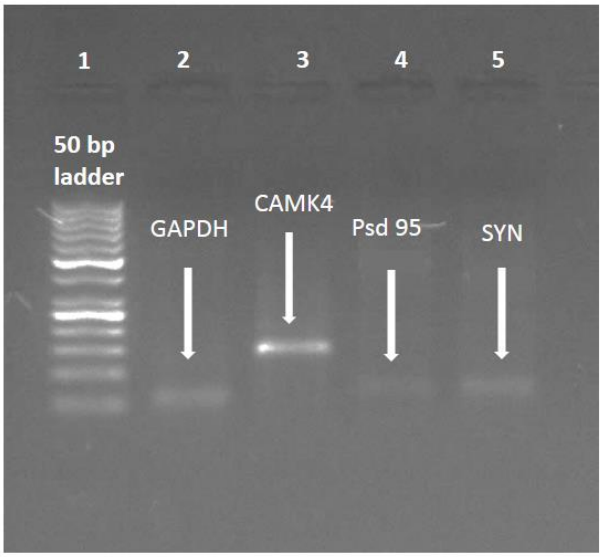

Figure 3.3: Primer optimization through gradient PCR.

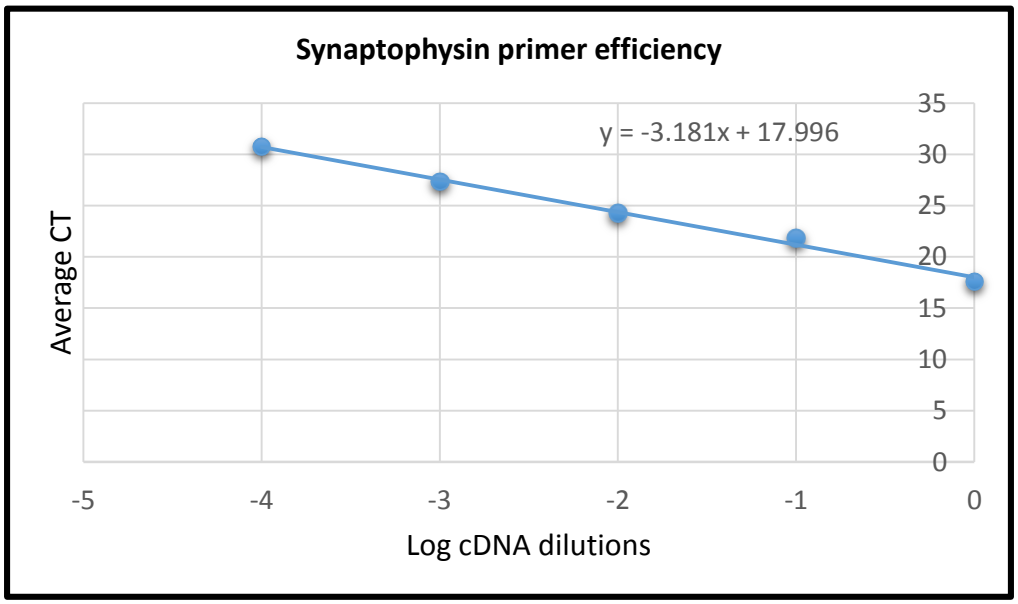

Figure 3.4: Graph representing primer efficiency of Synaptophysin gene

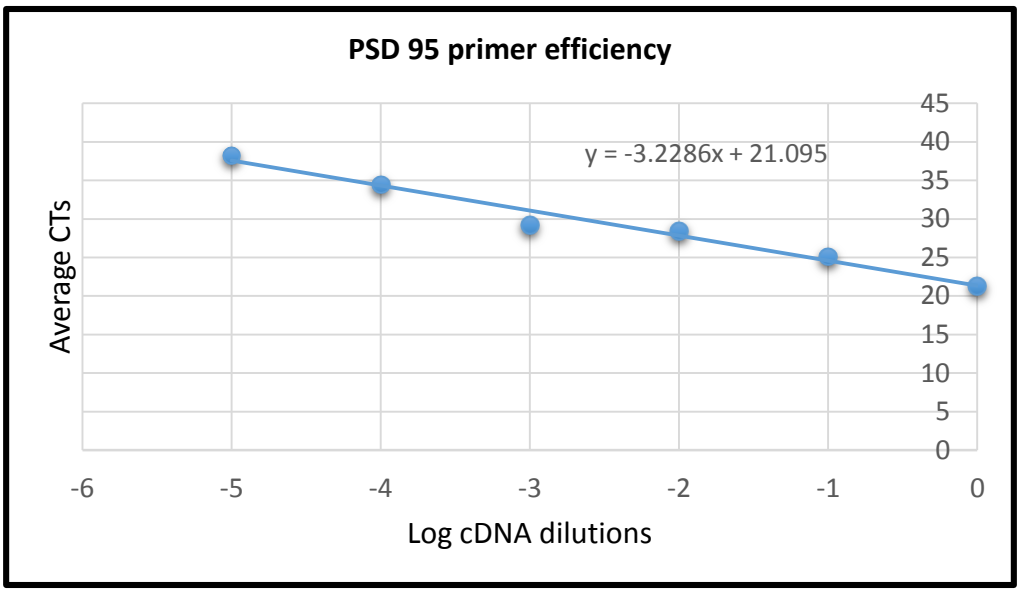

Figure 3.5: Graph representing primer efficiency of PSD 95 gene

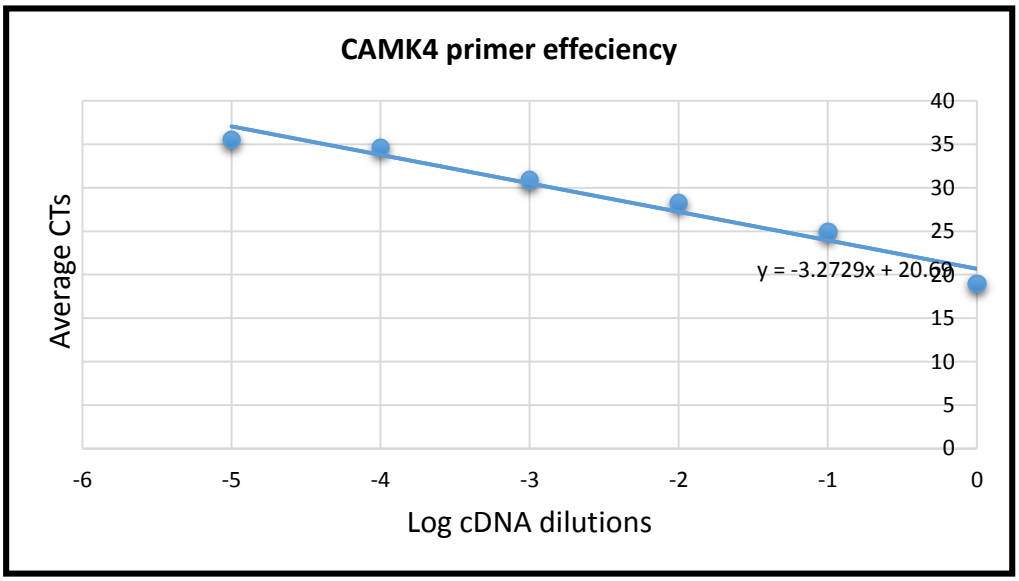

Figure 3.6: Graph representing primer efficiency of CAMK4 gene

3.1.5 Expression analysis of synaptophysin gene in cortex and hippocampus

Expression analysis of healthy control, metals+HFD group, metals+HFD+Shogoal (2mg/kg) and metals+HFD+Shogoal (12mg/kg) was accessed. qPCR analysis indicated that as compared to control group mice (1.0 ± 0.0), synaptophysin gene was significantly down regulated ($p< 0.01$) in metals+HFD group (0.07 ± 0.01) in the cortex region of the brain mice (Figure 3.7A) indicating neuronal impairment. metals+HFD+Shogoal (12mg/kg) mice group (0.88 ± 0.22) showed significant disease prevention ($p < 0.01$) indicating its pharmacological potential. metals+HFD+Shogoal (2mg/kg) (0.70 ± 0.26) mice group also showed significant up regulation with p-value ($p < 0.05$) compared to metals+HFD group (Figure 3.7A) suggesting that 2mg/kg dose also showed efficacy in preventing induction of neurotoxicity in the cortical region of the brain.

Similar results were observed in case of genetic expression of synaptophysin in the hippocampus (Figure 3.7B). A statistically significant down regulation was observed in the SYN expression in the metals+HFD group (0.15 ± 0.07) as compared to the normal control mice (1.0 ± 0.0), with p value of ($p < 0.01$) as shown (Figure 3.7B). Shogaol doses both 2mg/kg ((1.38 ± 0.40) as well as 12mg/kg (1.46 ± 0.14) were equally effective in up regulating the expression of SYN. Both doses showed a statistically significant up regulation in mice ($p < 0.001$).

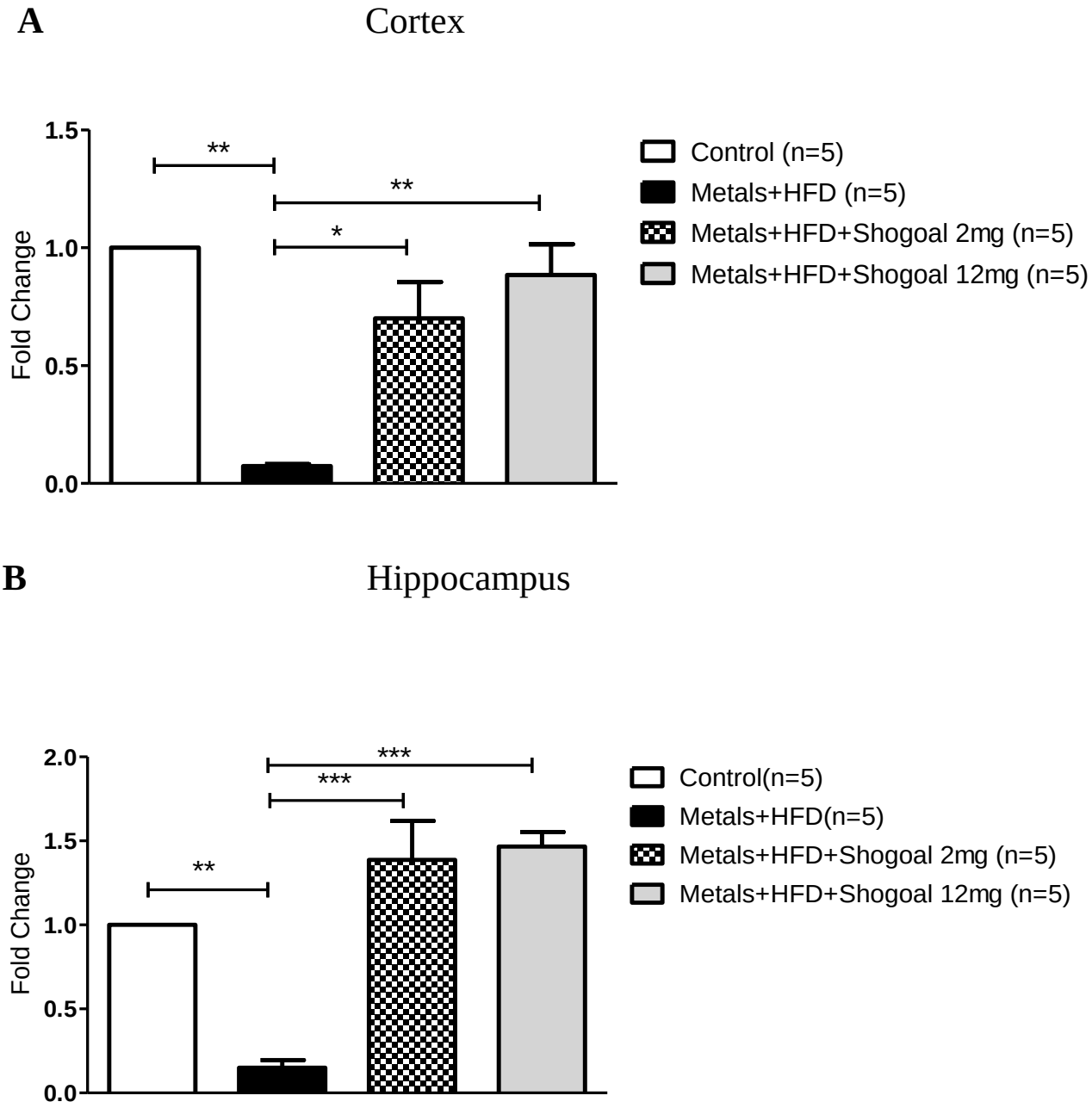

Figure 3.7: Histogram representing the relative expression of synaptophysin in cortex and hippocampus: The bar chart shows the relative expression of synaptophysin in control, metals+HFD, metals+HFD+Shogaol (2mg) and metals+HFD+Shogaol (12mg) in cortex (A) and hippocampus (B). The error bars represents the mean ± SEM for one-way ANOVA, followed by Bonferroni's Multiple Comparison test, *p= 0.05, **p=0.01 and ***p=0.001.

3.1.6 Expression analysis of PSD 95 gene in cortex and hippocampus

Expression analysis of healthy control, metals+HFD group, metals+HFD+Shogoal (2mg/kg) and metals+HFD+Shogoal (12mg/kg) was accessed (Figure 3.8B). Qualitatively genetic expression indicated a statistically significant decrease ($p < 0.05$) in the PSD 95 gene expression in metals+HFD (0.11 ± 0.02) group ($p< 0.01$) in the cortex region of the brain compared to the healthy control mice (1.0 ± 0.0) as shown (Figure 3.8A) indicating neuronal impairment due to metals and HFD consumption. Preventive mice models with intake of shogaol showed up regulation of PSD 95. metals+HFD+Shogoal (2mg/kg) (0.31 ± 0.32) mice group does showed an up regulation but data was not statistically significant. However, metals+HFD+Shogoal (12mg/kg) (0.97 ± 0.38) showed statistically significant ($p < 0.05$) up regulation compared to metals+HFD group (Figure 3.8A).

qPCR analysis indicated that PSD 95 gene was significantly down regulated in metals+HFD group (0.17 ± 0.08) with statistical significance of ($p< 0.01$) in the hippocampal region of the brain compared to the healthy control mice (1.0 ± 0.0) indicating neuronal impairment. Both the doses of Shogaol (2mg/kg, 12mg/kg) improved the expression of PSD 95 gene. metals+HFD+Shogoal (12mg/kg) mice group (1.4 ± 0.31) showed more significant disease prevention ($p< 0.001$) relative to metals+HFD+Shogoal (2mg/kg) (0.69 ± 0.28) mice as shown in (Figure 3.8B) when compared to metals+HFD group, suggesting that 12mg/kg dose is more efficient in preventing induction of neurotoxicity in the hippocampus.

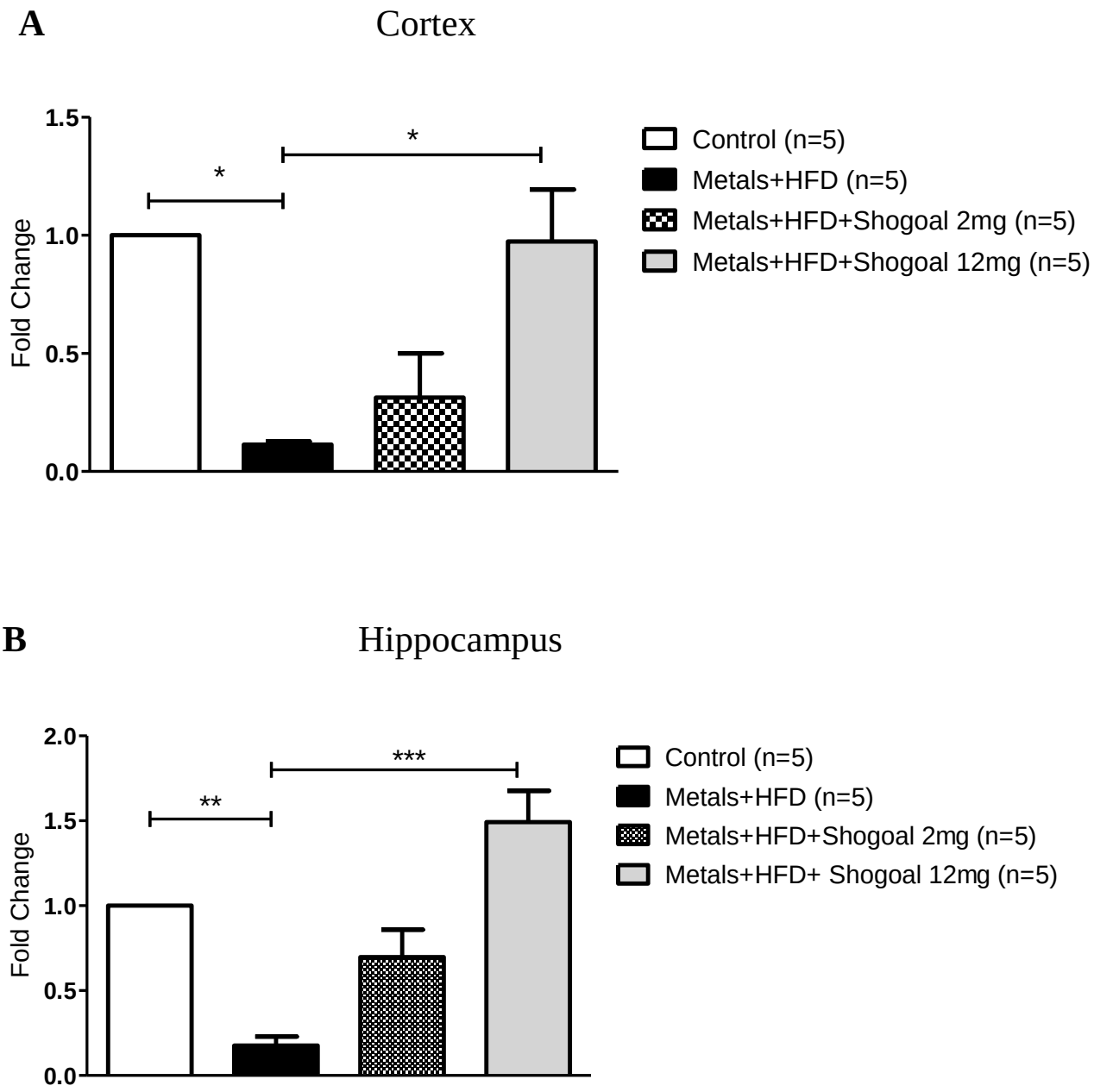

Figure 3.8: Histogram representing the relative expression of PSD 95 in cortex and hippocampus: The bar chart shows the relative expression of PSD 95 in control, metals+HFD, metals+HFD+Shogaol (2mg) and metals+HFD+Shogaol (12mg) in cortex (A) and hippocampus (B). The error bars represents the mean ± SEM for one-way ANOVA, followed by Bonferroni's Multiple Comparison test, *p= 0.05, **p=0.01 and ***p=0.001.

3.1.7: Expression analysis of CAMK4 gene in cortex and hippocampus

Expression analysis of healthy control, metals+HFD group, metals+HFD+Shogoal (2mg/kg) and metals+HFD+Shogoal (12mg/kg) was accessed (Figure 3.9A). qPCR analysis indicated that CAMK4 gene was significantly down regulated in metals+HFD group (0.33 ± 0.06) with statistical significance of ($p< 0.01$) in the cortex region of the brain compared to the healthy control mice (1.00 ± 0.0) indicating neuronal impairment (Figure 3.9A). Both the doses of shogaol (2mg/kg, 12mg/kg) improved the expression of CAMK4 gene. Metals+HFD+Shogoal (12mg/kg) mice (1.48 ± 0.48) group showed statistically more significant disease prevention ($p< 0.01$) as compared to metals+HFD+Shogoal (2mg/kg) mice (1.08 ± 0.25)group ($p<0.05$) when compared to metals+HFD group (Figure 3.9A), suggesting that 12mg/kg dose showed more efficacy in preventing induction of neurotoxicity in the cortical region of the brain .

In the hippocampal regions of brain, qPCR analysis was carried out in all the four groups. CAMK4 gene was down regulated in metals+HFD group (0.75 ± 0.17) compared to the healthy control mice (1.00 ± 0.0) but the down regulation was not statistically significant, indicating chances of neuronal impairment (Figure 3.9B). Shogaol showed an evident up regulation of CAMK4 gene. However, this up regulation was statistically more significant in metals+HFD+Shogoal (2mg/kg) (1.4 ± 0.35) mice group ($p<0.001$) as compared to metals+HFD+Shogoal (2mg/kg) (2.03 ± 0.19) mice group ($p<0.05$) compared to metals+HFD group (Figure 3.9B).

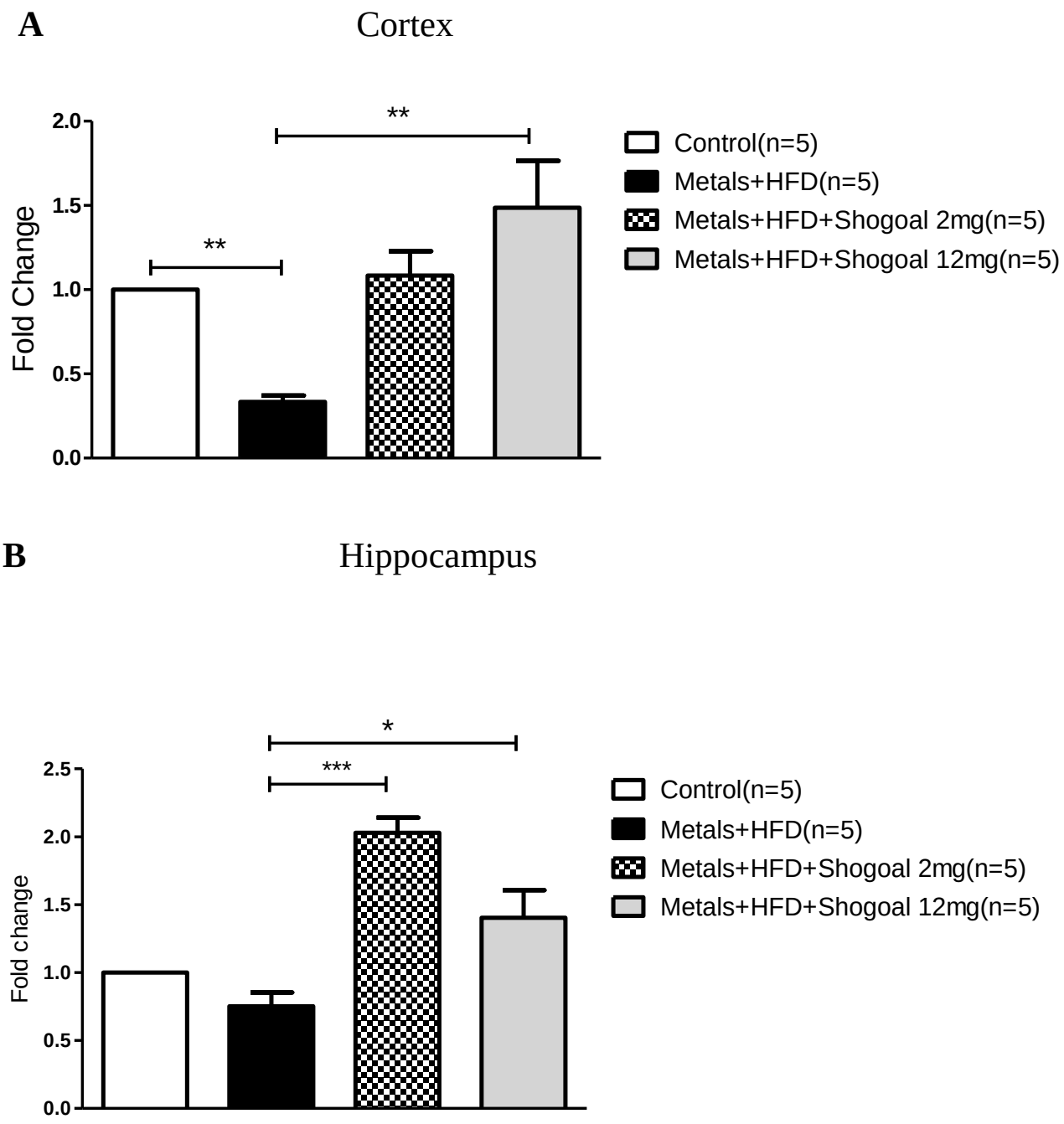

Figure 3.9: Histogram representing the relative expression of PSD 95 in cortex and hippocampus: The bar chart shows the relative expression of PSD 95 in control, metals+HFD, metals+HFD+Shogaol (2mg) and metals+HFD+Shogaol (12mg) in cortex (A) and hippocampus (B). The error bars represents the mean ± SEM for one-way ANOVA, followed by Bonferroni's Multiple Comparison test, *p= 0.05, **p=0.01 and ***p=0.001.

3.2 Histological examination of brain regional tissues

Neuro-degeneration was observed in different regions of brain along with synaptic impairment. Both hippocampus and cortex were highly affected due to exposure to high fat diet and metals. Cresyl violet staining showed marked appearance of shrunk neurons and a decrease in the neuronal cell count. However, shogaol appeared to be retaining the morphology of different brain regions.

3.2.1: histological assessment of cortex

Cortical brain tissues of mice were observed within motor cortex and somatosensory cortex regions at 10X and 40X magnification. Control group showed intact morphology within different layers of brain (layer 1 to layer 6) as shown (Figure 3.10A) at 10X, while intact neuronal density was observed in cortex at 40X magnification (Figure 3.10B). In Metals+HFD group, there is visible disruption of tissue with no clear distinction of layers due to damage as indicated in (Figure 3.10). Neuronal density also decreases in the cortex due to high fat and metals consumption. In 3rd group of Metals+HFD+Shogoal (2mg/kg), shogaol has shown to prevent the destruction and neuronal degeneration to a greater extent as the cell density is comparatively greater that Metals+HFD group and also structural morphology is less distorted, while mice with Metals+HFD+Shogoal (12mg/kg) showed greater prevention from disruption due to high fat and metals consumption. Moreover the layers of cortex were also distinct and distinguishable at 10X magnification. 40X showed intact neuronal cells with visible neuronal density indicating that 12mg/kg was the effective dose in preventing the effect of high fat and metals (Figure 3.10B).

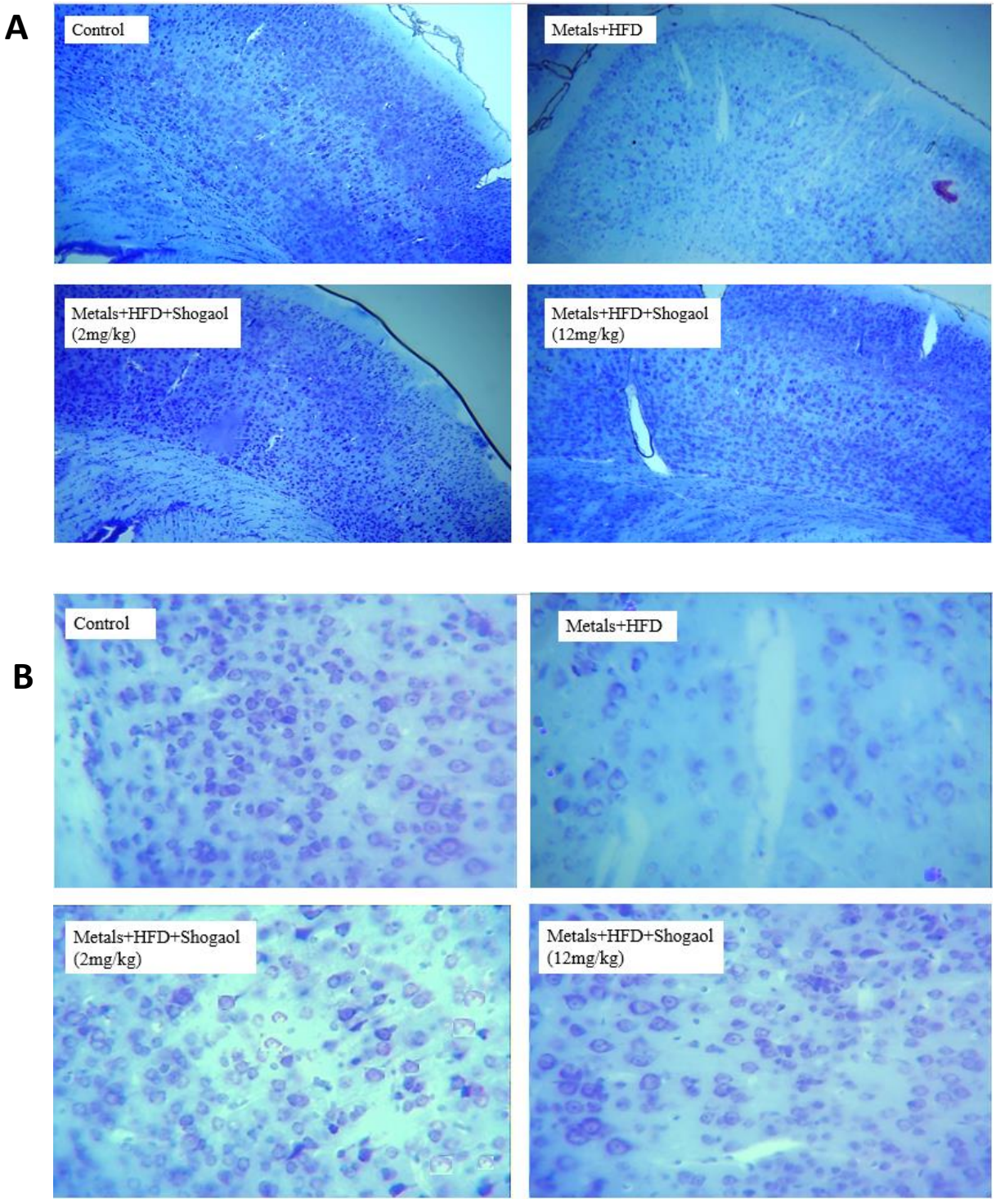

Figure 3.10: Cresyl violet stained sections of hippocampus: Figure represented the sections of cortex at 10X (A) and 40X (B) in control, metals+HFD, metals+HFD+Shogaol (2mg) and metals+HFD+Shogaol (12mg).

3.2.2 Histological assessment of hippocampus

All the four regions of hippocampus i.e.; dendate gyrus, CA1, CA2, and CA3 were observed through nissl staining at 10X and 40X magnification. C shaped hippocampus was clearly observed in control group mice showing intact regions and properly confined neuronal cells. Distribution of neurons was uniform. In metals+HFD group, there was scattering of neurons as well as shrinkage of structure of hippocampus, indicating the signs of neurodegeneration, evident at 10X magnification (Figure 3.11A). Neuronal cell density was decreased in all the sections of hippocampus in metals+HFD group and neuronal cell death was also present (Figure 3.11A). In metals+HFD+Shogoal (2mg/kg), cells showed less destruction and lesser shrinkage of hippocampus, indicating that Shogaol provided therapeutic effect and prevented the destruction due to metals and high fat. However, 4th group mice with metals+HFD+Shogoal (12mg/kg), showed structural morphology similar to the healthy controls. Neuronal cells were intact and neuronal cell density was similar to healthy controls as shown at 40X magnification (Figure 3.11B). It indicated that 12mg/kg of Shogaol was efficient in preventing the damage due to consumption of Shogaol and metals.

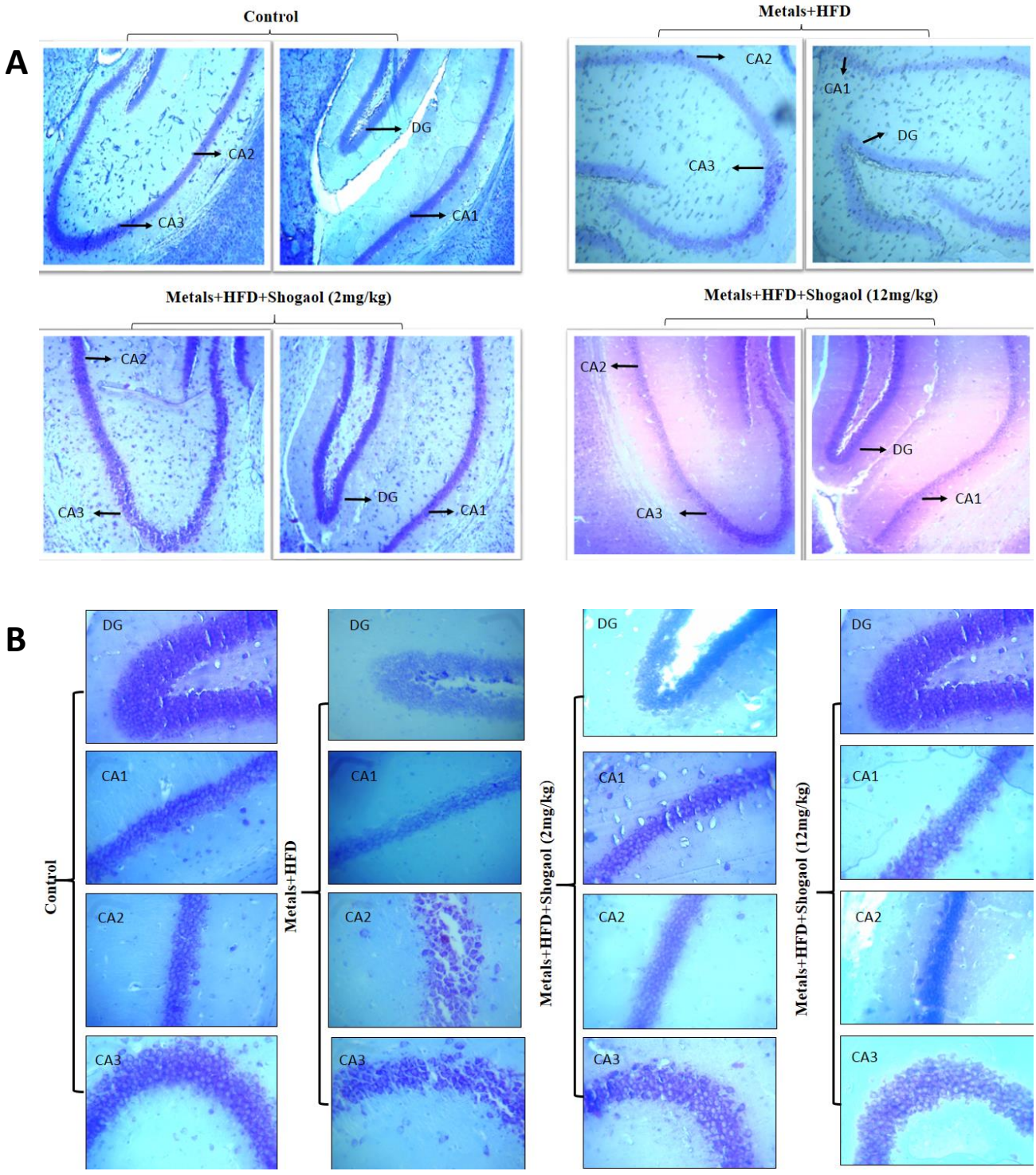

Figure 3.11: Cresyl violet stained sections of hippocampus: Figure represented the sections of hippocampus at 10X (A) and 40X (B) in control, metals+HFD, metals+HFD+Shogaol (2mg) and metals+HFD+Shogaol (12mg).

3.3 Determination of cell count

Cresyl violet staining was carried out to detect the cell number quantitatively in cortex as well as hippocampus at 10X and 40X respectively.

3.3.1 Neuronal cell count in cortex

Cortical region at motor and somatosensory region was quantitatively determined at 10X magnification. It was shown that control group mice showed a regular pattern of neuronal cell count in molecular layer 1(5.62±0.47), extra granular and pyramidal layer 2 and 3 (16.94±3.50), inner granular layer 4 (13.44±2.06), ganglionic layer 5 (12.69±1.97) and multiform layer 6 (13.06±0.85) as shown (Figure 3.12 A-B), (figure 3.13 A-B) and (Figure 3.13) . In metals+HFD group a significant decrease in cell count was observed in all the layers as layer 1 (1.75±0.79; $p<0.01$), layer 2-3 (7.18±2.49; $p<0.01$), layer 4 (7.61±1.78; $p<0.01$), layer 5 (8.25±1.06; $p<0.05$) and in layer 6 (7.87±3.29; $p<0.05$) as shown (Figure 3.12.3.13.3.14) .Data represented significant reduction of neuronal cells, indicating occurrence of neurodegeneration. In mice with metals+HFD+Shogoal (2mg/kg) and metals+HFD+Shogoal (12mg/kg), there was clear indication of retention of cell count due to shogaol intake. Shogaol provided the protective effect and prevented cell death and showed statistically significant data in all the layers as layer 1,2-3,4,5 and 6 as (6.31± 1.76; $p<0.001$), (12.56±3.98), (11.19±2.68), (12.63±2.42; $p<0.05$) and (12.81±1.32; $p<0.05$) respectively. metals+HFD+Shogaol (12mg/kg) affected the cell count more efficiently and showed statistically significant data (Figure 3.12,3.13 and 3.14). At this dose Shogaol showed its potential in preventing the effects of HFD and metals and retained the cell number in layer 1,2-3,4,5 and 6 as (5.56±1.24; $p<0.01$), (15.56±1.93; $p<0.05$), (12.81±1.42; $p<0.05$), (13.50±1.35; $p<0.01$) and (12.81±0.85; $p<0.05$).

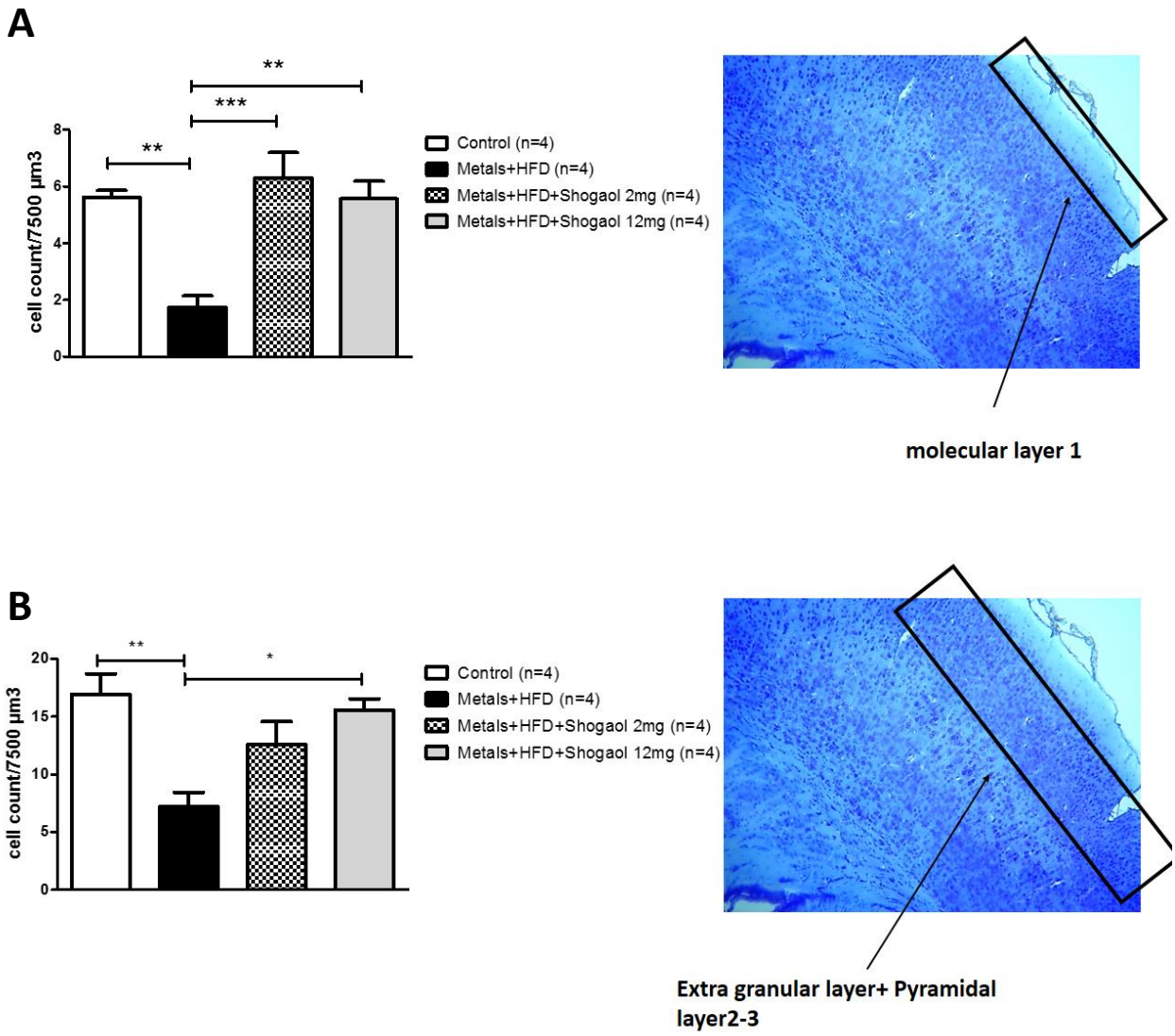

Figure 3.12: Graph representing cell number in cortical layers: Bar graphs represents the cell number in Molecular layer (A) and Extra granular and pyramidal layer (B) in Control, metals+HFD, metals+HFD+Shogaol (2mg) and metals+HFD+Shogaol (12mg) of cortex. The error bars represents the mean ± SEM for one-way ANOVA, followed by Bonferroni's Multiple Comparison test, *p= 0.05, **p=0.01 and ***p=0.001.

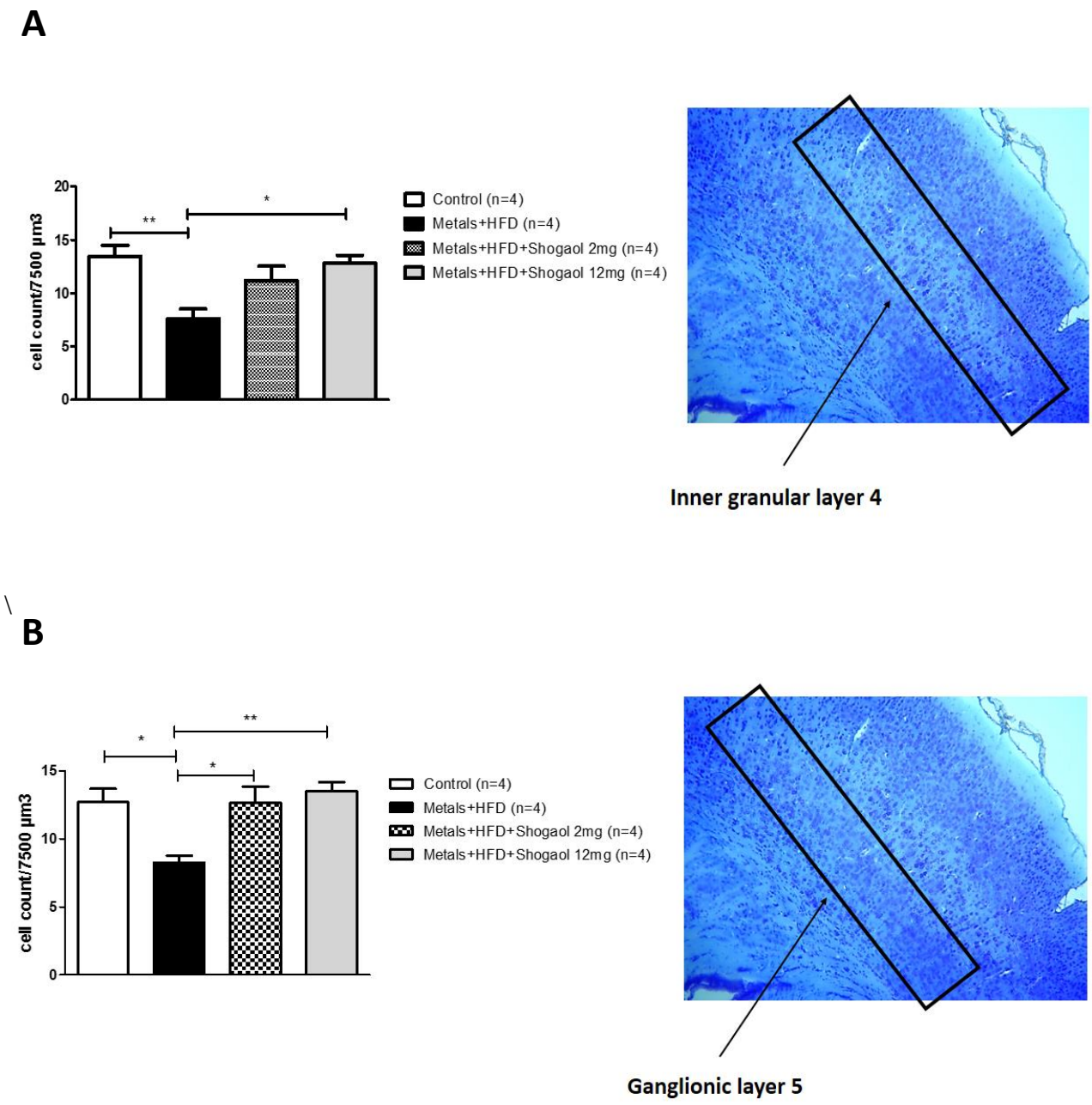

Figure 3.13: Graph representing cell number in cortical layers: Bar graphs represents the cell number in Inner granular layer (A) and Ganglionic layer (B) in control, metals+HFD, metals+HFD+Shogaol (2mg) and metals+HFD+Shogaol (12mg) of cortex. The error bars represents the mean ± SEM for one-way ANOVA, followed by Bonferroni's Multiple Comparison test, *p= 0.05, **p=0.01 and ***p=0.001.

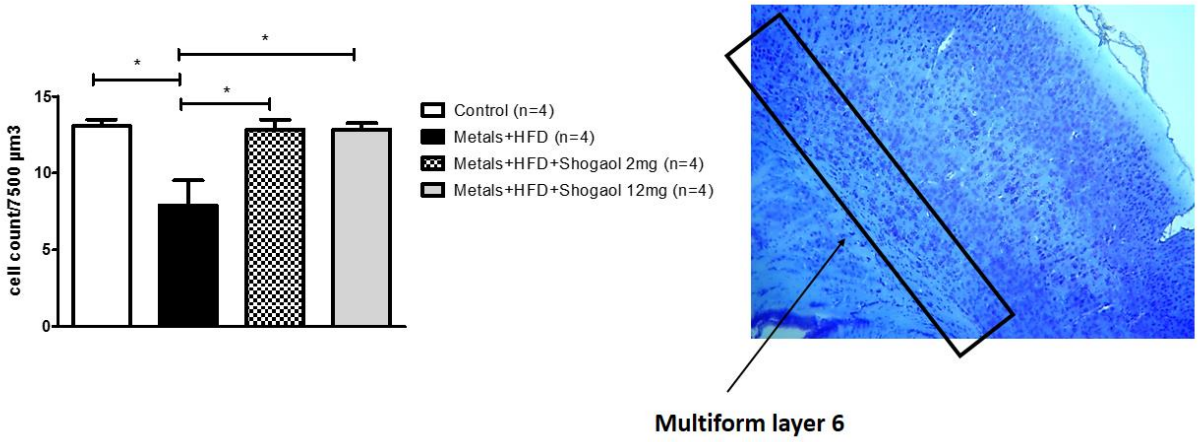

Figure 3.14: Graph representing cell number in cortical layers: Bar graphs represents the cell number in multiform layer in control, metals+HFD, metals+HFD+Shogaol (2mg) and metals+HFD+Shogaol (12mg) of cortex. The error bars represents the mean ± SEM for one-way ANOVA, followed by Bonferroni's Multiple Comparison test, *p= 0.05, **p=0.01 and ***p=0.001.

3.3.2 Neuronal cell count in hippocampus

Quantitative neuronal cells were determined in different sections of C shaped hippocampus as dendate gyrus, CA1, CA2 and CA3. It was shown that control group mice showed a regular pattern of neuronal cell count in dendate gyrus (7.50 ±0.40), CA1 region (6.68±0.62), CA2 (6.06±1.08), and CA3 (5.06±.12). In metals+HFD group a significant decrease in cell count was observed in all sections as dendate gyrus CA1 (3.25±0.40; $p<0.001$), CA2 (2.93±0.37; $p<0.001$), CA3 (2.06±0.65; $p<0.001$), (3.56±0.12; $p<0.001$). Data represented significant reduction of neuronal cells, indicating occurrence of neurodegeneration. In mice with metals+HFD+Shogoal (2mg/kg) and metals+HFD+Shogoal (12mg/kg), there was clear indication of retention of cell count due to shogaol intake. Shogaol provided the protective effect and prevented cell death and showed statistically significant data in all the sections of DG, CA1, CA2 and CA3 as (5.75± 0/.79; $p<0.01$), (5.68± 1.06; $p<0.01$), (4.93± 0.23; $p<0.05$), and (4.75± 0.35; $p<0.001$) respectively.

Metals+HFD+Shogaol (12mg/kg) affected the cell count more efficiently and showed statistically significant data. At this dose shogaol showed its potential in preventing the effects of HFD and metals and retained the cell number data in all the sections of DG, CA1, CA2 and CA3 as (6.32± 0.85; $p<0.001$), (5.31± 0.23; $p<0.01$), (5.47± 0.78; $p<0.01$) and (4.85± 0.31; $p<0.001$) respectively.

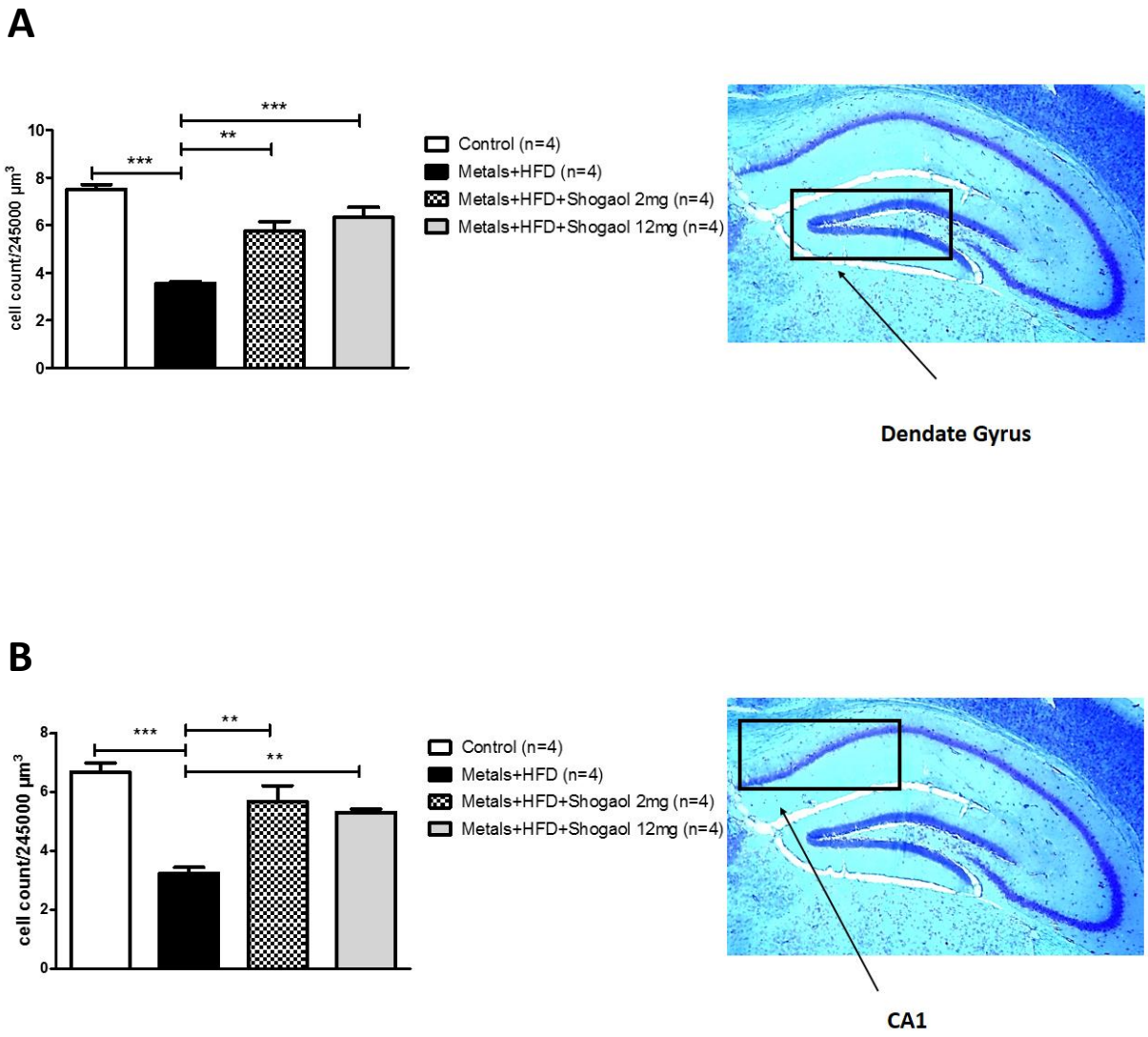

Figure 3.15: Graph representing cell number in C shaped hippocampal regions: Bar graphs represents the cell number in dendate gyrus (A) and CA1 (B) in control, metals+HFD, metals+HFD+Shogaol (2mg) and metals+HFD+Shogaol (12mg) of hippocampus. The error bars represents the mean ± SEM for one-way ANOVA, followed by Bonferroni's Multiple Comparison test, *p= 0.05, **p=0.01 and ***p=0.001.

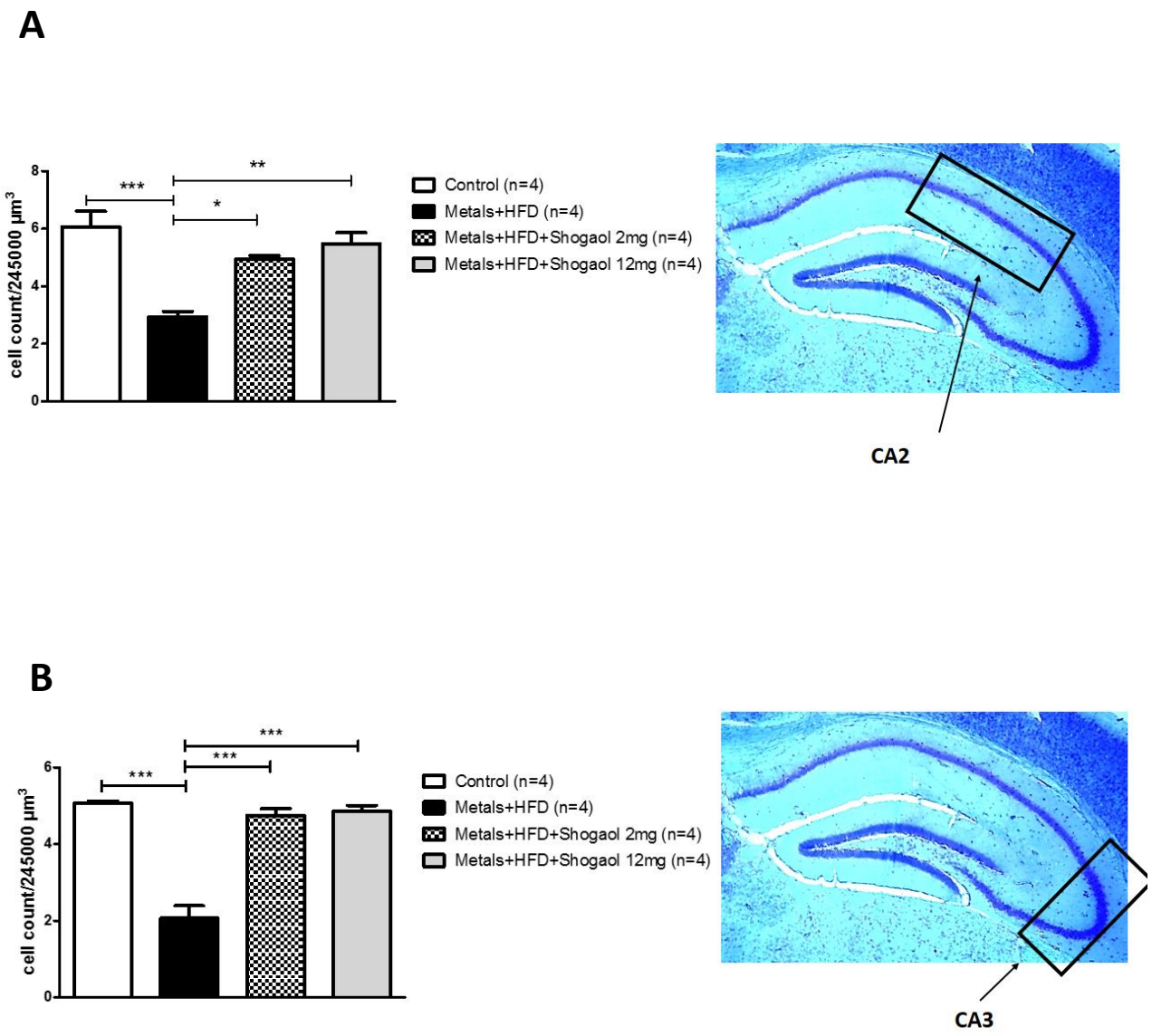

Figure 3.16: Graph representing cell number in C shaped hippocampal regions: Bar graphs represents the cell number in CA2 region (A) and CA3 region (B) in control, metals+HFD, metals+HFD+Shogaol (2mg) and metals+HFD+Shogaol (12mg) of hippocampus. The error bars represents the mean ± SEM for one-way ANOVA, followed by Bonferroni's Multiple Comparison test, *p= 0.05, **p=0.01 and ***p=0.001

DISCUSSION

Brain of higher organisms is composed of complex networks of neurons which constitute the central nervous system. These neurons are not randomly distributed but composed of specific network, pathway and pattern, which work together to carry out many functions. Altogether this regular arrangement makes the entire brain structure. These networks are widely distributed in different regions of brain and carry out particular functions. Not only the structure of neuron, but the location as well as concentration is also crucial in processing the brain related functions of body such as learning, memory formation, long term memory formation, motor coordination, emotion related abilities and language etc. focusing on learning and memory processes, then the two regions which are important in this aspect are hippocampus and cortex. Interplay of both these regions of brain help in regulating the process of learning and memory as well as storage of memory (Jin and Maren, 2015). Previous data suggested that cortex and mainly motor cortex maintains the cognitive functions and especially memory formation (Moscovitch, 1992), while hippocampus is involved in learning and memory as well as neuronal plasticity for long term potentiation, as was evident from the famous study of H.M. (Scoville and Milner, 1957).

So basically, the memory formed is based on the previous experiences as well as upon the information stored in the cortical as well as hippocampal region. Consolidation of memory occurs and in this process both hippocampus and cortex are involved as hippocampus mainly guides the organizational process and transfer it to neocortex (McGaugh, 2000). Hippocampus is the main region of brain involved in memory formation. It is involved in formation of both instant memory as well as converting this short memory into long term memory by strengthening of neuronal connections and hence hippocampus is the main

region where permanent storage of information and memory takes place (Scoville and Milner, 1957). Hippocampus is composed of different regions and signal flows takes place sequentially in each region. Firstly, it comes to the CA1 region, moving to CA2 and ultimately CA3. Each region shows different range of processes as CA1 responds to instant learning and memory while CA2 responds to long term potentiation process while CA3 processes the neuronal plasticity (Sydow et al., 2011).

Clean drinking water is essential for the normal homeostasis of body and ground water Is the biggest source of drinking water in Pakistan, but clean and contamination free drinking water is getting scare and unavailable to humans that is why they are forced to use the contaminated water. Metals contamination is one of the biggest problem of current era. Toxic metals such as lead, arsenic, cadmium, aluminum and mercury are present in drinking water. The main source of these metals is the industrial zone, agricultural zone as well as mining. Such metals en route to human body have no useful role but only cause damage to the organs. (Waseem et al., 2014). Metal neurotoxicity is caused by many ways, such as oxidative stress, inhibition of calcium signaling pathway, interfering with synaptic process, causing inflammation as well as disrupting the blood brain barrier (Caito and Aschner, 2015).arsenic is toxic, carcinogenic and also involved in causing cognitive impairment. Lead is reported to be directly accumulating in the hippocampus and interfere with the process of synaptic plasticity and also a risk factor for causing metabolic disorders (Papanikolaou et al., 2005). Aluminium is the main metal involved in risk factor for Alzheimer disease, and oral administration of aluminium in water has been reported to be the established model in inducing dementia models have been reported (de Jesús Ramírez-Altamirano et al., 2012).

High fat diet intake is another major concern for developing cognitive decline along with other metabolic disorders. These dietary factors are also having a role in synaptic process and function of neurons involved in learning and memory. (Granholm et al., 2008). Chronic exposure to high fat diet causes direct insulin resistance as well as oxidative stress, which ultimately leads to the damage to the memory formation process in hippocampus (Alzoubi et al., 2018).

Certain plants are known to have medicinal importance such as turmeric powder, thymus tea, rosemary etc. as these plants possesses certain compound which as useful such as phenols, terpenoids phytochemicals (Achilonu et al., 2018). Ginger is the plant, widely used across the world as spice. It is known to have some potential as anti-tumor, anti-cancer, anti-diabetic as well as neuroprotective (Sapkota et al., 2019). Previous data reported that shogaol showed neuroprotective effect in both in vitro and in vivo testing. It is also reported that shogaol aids in protecting the neuronal cells from the damage caused by beta plaques

(Kim and Kim, 2004). It was also studied earlier that shogaol protected cholinergic neurons from reactive oxygen species in hippocampal neuronal (Shim and Kwon, 2012).

A lot of research has been done on metals and high fat diet and it has been reported that exposure to both metals as well as high fat diet consumption causes impairment to synapses as well induces neurodegeneration. However, either their combination (Metals + HFD) cause synaptic impairment and neurodegeneration or not and to which extent, is yet to be explored and characterized. Shogaol has known to have neuroprotective effect but the underlying mechanism of its pharmacological effect is remained to be elucidated.

There is no such study on the effect of shogaol in the regulation of genes involved in learning, memory and synaptic plasticity.

The present study was conducted to investigate the effect of Metals and HFD in combination on synaptic impairment and learning and memory formation in mice model as well as to assess the pharmacological potential of shogaol in preventing the effect of metals and HFD. Four groups of animals were selected. Of which one was control receiving normal diet and distilled water over the course of study. One group received Metals and HFD in combination. Remaining two groups received Metals and HFD along with Shogaol at two different concentrations, (2mg and 12mg). In order to identify the protective effect of shogaol, gene expression analysis of synaptic genes was carried out to identify that either shogaol is capable of improving short term learning and memory or long term potentiation or both. Neurodegeneration induced by the metals and HFD and prevented in Metals and HFD and shogaol group was also examined both qualitatively and quantitatively through histological examination and cell counting process respectively.

Previously it was reported that HFD reduces the expression of synaptic genes as well as disrupt the neuronal morphology and cognition (Nam et al., 2017) as well as HFD induces obesity which causes insulin resistance and ultimately leading to effects on synaptic plasticity (Liu et al., 2015). Previously it was also reported that metals neurotoxicity leads to the synaptic impairment (Sadiq et al., 2012). But there was no approachable data to investigate the combined effect of metals and HFD on synaptic genes. In the current study synaptic genes were investigated (synaptophysin, PSD 95 and CAMK 4). All the genes showed the down regulation in response to Metals and HFD exposure. Synaptophysin

gene expression was tested in both the hippocampus and cortex and in both the regions, it showed a significant down regulation in both the regions involved in learning and memory. PSD 95 gene also showed significant down regulation in response to Metals and HFD, but in this case the down regulation was more significant in hippocampus as compared to cortex. The down regulation of both these genes depicted that HFD and Metals decreases the learning and memory process. While the expression of CAMK 4 gene was also investigated and the results showed that its expression was significantly down regulated in cortical region but there was no statistical significance in hippocampus. Since CAMK4 is a mediator of transcriptional activation of genes involved in synaptic plasticity so it is deduced form the results that HFD and Metals do not showed an obvious effect in hindering the synaptic plasticity. Hence, it can be said that metals and HFD are effective in preventing learning and memory but nor long term memory. The group receiving Shogaol along with Metals and HFD also showed significant results. It was shown that Shogoal (2mg) showed an increase in expression of Synaptophysin, but the results were not significant for PSD 95 and again CAMK 4 showed varied results with an excessive up regulation in hippocampus. Shogaol (12mg) showed promising results for all the three genes, as expression was normalized to healthy controls indicating 12mg prevented the effect of Metals and HFD and maintained the expression of genes.

It has been reported in literature that High fat diet consumption induces obesity which ultimately leads to neurodegeneration. Neuronal cell number decrease and neurotoxicity is induced (Sah et al., 2017) and also causes neuro-inflammation and cognitive decline all resulting in altered morphology of brain (Duffy et al., 2019). Along with synaptic impairment, metal and HFD also induces neurodegeneration and shrinkage of brain

regions. Control groups showed intact structural morphology and division of cortical layers while as well as properly identifiable C shaped hippocampus, while the group of mice with Metals and HFD showed disturbed layers of cortex and shrunken C shaped hippocampus. Shogaol (2mg) showed some prevention of neurodegeneration and retained shape of hippocampus and lesser shrinkage, while Shogaol 12mg showed structural morphology similar to healthy controls and compactly packed neuronal cells.

Quantitatively cell count also decreases due to metals and HFD exposure, it was previously reported in a study that intake of metals and high fat diet significantly reduced the cell count in both hippocampus regions and cortex (Iqbal and Ahmed, 2019). In the present study, quantitative cell count was carried out in hippocampal regions as well as in the layer of cortical region at motor cortex and somatosensory region. It was shown that metals and HFD groups showed significant reduction of neurons while Shogaol prevented this damage by maintaining the neuron count constant. So form this study it can be concluded that Shogaol can be used as an effective natural compound in preventing neuronal damage as well as in treating the neurodegenerative diseases.

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