Evaluation of Therapeutic Effects of Mecobalamin against Arsenic induced Neurotoxicity in Rats

Master of Science in Healthcare Biotechnology

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Healthcare Biotechnology

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Sameen Fatima

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ABBREVIATIONS

CNS	Central Nervous System
DG	Dentate Gyrus
CA1	Cornu ammonis 1
CA3	Cornu ammonis 3
BBB	Blood Brain Barrier
Cbl	Cobalamin
MeCbl	Mecobalamin
PSD-95	Post-Synaptic Density Protein-95
SYN	Synaptophysin
SNAP-25	Synaptosomal-associated protein-25
CAMK-IV	Calcium/calmodulin-dependent protein kinase IV
Nrxn	Neurexin
Nlgn	Neuregulin
Shank1	SH3 And Multiple Ankyrin Repeat Domains 1
Shank 2	SH3 And Multiple Ankyrin Repeat Domains 2
SOD	Superoxide dismutase
CAT	Catalase
GPx	Glutathione peroxidase
ROS	Reactive oxygen species

OS	Oxidative stress
TNF-α	Tumor necrosis factor- α
CRP	C- Reactive protein
MBP	Methylation dependent myelin basic protein
ATSDR	Agency for Toxic Substances and Disease Registry
WHO	World Health Organization
AD	Alzheimer's disease
DMA	Dimethylarsonic acid
MMA	Monomethylarsonic acid
COX-2	Cyclo-oxygenase-2
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
rpm	revolution per minute
NBT	Nitro blue tetrazolium
DTNB	Dithiobisnitrobenzoic acid
H2O2	Hydrogen peroxide
dNTPs	Deoxynucleotide triphosphate
RT	Reverse Transcriptase

ABSTRACT

Arsenic contaminated water affected large population of people worldwide and is associated with innumerable disorders of nervous system. Arsenic-induced neurotoxicity leads to disturbance in memory and learning and synaptic plasticity. Cobalamin (Cbl), a water-soluble vitamin, possess positive effect on synaptic plasticity mediated memory and learning in many reported studies. It also possess anti-inflammatory properties while its deficiency is linked with neuronal apoptosis. The study was conducted for examining the cobalamin homologue mecobalamin (MeCbl) effects on the several significant genes expression involved in synaptic plasticity along with antioxidant enzymes activity i.e glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) along with neuronal apoptosis. Sodium arsenate (80 mg/kg) an inducer of neurotoxicity administered along with MeCbl treatment for the period of two months at two selected doses (5 mg and 10 mg per kg of diet) in rats. Results indicated that arsenic treatment induced more significant ($p < 0.001$) downregulation in the expression of Synaptophysin (Syn), Post-Synaptic Density Protein 95 (PSD-95), SH3 And Multiple Ankyrin Repeat Domains 1 (Shank 1), SH3 And Multiple Ankyrin Repeat Domains 2 (Shank 2), Neurexin 2 (Nrxn 2), Neuroligin 1 (Nlgn 1), Kalirin and Calcium/calmodulin-dependent protein kinase IV (CAMK-IV) and less significant ($p <0.01$) downregulation in the expression of Nrxn 1 and Shank 1. The expression of Neuregulin 2 (Nlgn 2) and Synaptosomal associated protein (SNAP-25) was slightly reduced ($p < 0.05$) while all the study groups showed similar expression of Homer. Arsenic treatment induced oxidative stress leading to substantial decrease ($p < 0.001$) in antioxidant activity Histopathological examination revealed a significant decrease in cell density in DG ($p < 0.001$) and CA3, CA1 ($p < 0.01$) regions of

hippocampus after arsenic treatment. Simultaneous treatment with MeCbl (10 mg) resulted in positive shift in the expression of SYN, Shank 1, Shank 2, Nrxn 2, PSD-95, Kalirin and CAMK-IV while the expression of Nlgn 2, SNAP-25 and Nlgn 2 was lower than arsenic treated group. There was increased activity of antioxidant enzymes (SOD, GPx and CAT) in rats with MeCbl treatment relative to arsenic treated rats along with less significant CAT activity in MeCbl (5 mg) treated rats. DG and CA3 regions of the hippocampus showed increased cell count in both MeCbl treated group rats but only MeCbl (10 mg) treated rats were able to recover the cell density in CA1 region of hippocampus. The findings of present study suggest that MeCbl (10 mg/kg) can be an effective option to reverse the changes in synaptic plasticity, histopathology and antioxidant status induced by arsenic.

CHAPTER 1

INTRODUCTION

1.1. Neurotoxicity

Any toxic influence on the chemistry, anatomy and physiology of the nervous system during developmental or growth period persuaded by any biological or biochemical (internal or external) stimuli is characterized as neurotoxicity. The alterations in the morphology include pathology with neuronal loss, functional or structural deficit in axons leading to neuron degeneration, glial cells myelin sheath loss and many other gliopathies. Even trifling or transitory changes in function and structure have adverse effects (Thakur et al., 2021).

Cerebral palsy, attention deficit disorder, autism and mental retardation have association with neurotoxicity induced by heavy metals (Jaishankar et al., 2014; Lee et al., 2018). People working in industries are exposed to the metals. Therefore, the amount of metal exposure is controlled at the acceptable limit by governmental organizations for the safety of workers. Keeping aside the role of heavy metals in normal physiology, high level of metal exposure for long or short time period results in substantial health risks and susceptibility of central nervous system (CNS) is very high for exposure to metals. Essential metalloproteins produced by accumulation of heavy metals in brain are indispensable for energy homeostasis and health of neurons but excessive accumulation of essential or nonessential metals leads to severe neurological complications (Caito et al., 2015; Chen et al., 2016).

1.2. Arsenic induced Neurotoxicity

Neurotoxicity persuaded by arsenic is conjectured by modifications in physical mechanisms comprising higher level of reactive oxygen species (ROS) with increase in lipid peroxides, oxidative pressure and subsiding the enzymatic activities of antioxidants such as glutathione peroxidase (GPx) and superoxide dismutase (SOD) (Garza-Lombó et al., 2019).Arsenic induced neurotoxicity symptoms consisting of unresponsiveness of the limbs, subclinical nerve injuries containing peripheral neuropathies, attenuation in muscle power, prickly sensation, restlessness and encephalopathy, unusual visual sensations, confusion, mental sluggishness, disorientation, distorted vision, loss of taste and hearing are reported in copper smelting industries workers (Chandravanshi et al., 2019).

Main factors involved in neurotoxicity of heavy metal include oxidative stress (OS), impairment of neurotransmission, bio-physics alterations in membranes and deregulation of cell signaling (Andrade et al., 2017) and cause lipid peroxidation with the generation of hydrogen peroxide, singlet oxygen and hydro peroxides leading to depletion of antioxidant enzymes and this whole phenomenon is known as OS and upward thrust in ROS results in a number of neural perturbations comprising boosted permeability of blood–brain barrier (BBB) and changes transmission across the synapsis (Figure 1.1). Principle mechanism of arsenic induced neurotoxicity includes its interface with assemblies of protein (sulfhydryl and thiol group) associated with functional disturbance of around 200 enzymes. Arsenic could also substitute molecules i.e. phosphate in various living pathways subsequently leading to rehabilitated pathways (Singh et al., 2017).

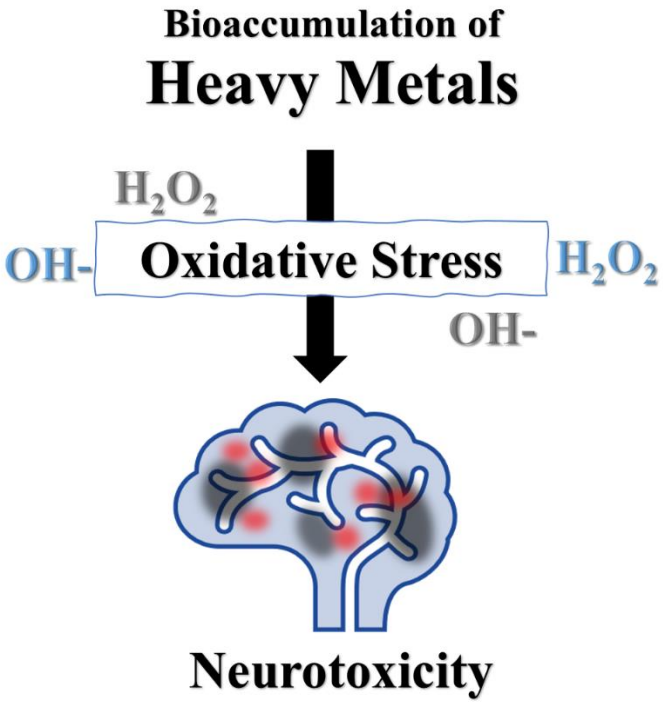


Figure 1.1. Mode of action of heavy metals

Animal research has shown substantiation for the arsenic aptitude to enter brain parenchyma after crossing BBB. Brain becomes progressively vulnerable to free radicals damage due to arsenic exposure consequently resulting in unusual neuronal death (Sun et al., 2017). Striatum and hippocampus are the sites in CNS for accumulation of arsenic (Tyler and Allan, 2014). Likewise, to ROS factors, the inflammatory peacekeepers including interleukins, tumor necrosis factor (TNF)-α, and C-reactive protein (CRP) are also raised in the pregnant woman plasma due to environmental contamination of arsenic (Azizieh et al., 2016; Stigger et al., 2013). Antioxidants as valuable and effective mediators act against neurotic ailments in body organs triggered by OS (Luo et al., 2012). He et al,

(2021) publicized that the myelin sheath loss is induced by arsenic in rats because it prevents the production of methylation-dependent myelin basic protein (MBP).

Arsenic neurotoxicity comprises paralysis, symmetrical peripheral neuropathy, diminished muscle power, misperception, sleep disorders, suspicious ideation, delirium, confusion, emotional liability, severe nervousness, blurred vision, loss of taste and hearing, impairment of higher neural functions such as concentration, learning and recent memory (Mochizuki, 2019; Luo et al., 2012). A variety of research have revealed susceptibility of cortex, corpus striatum and hippocampus to arsenic toxicity which might be accompanying with neurochemical and behavioral anomalies (Ramos-Chávez et al., 2015; Tripathi et al., 2022). Arsenic presence in the human body is closely associated with organ malfunction and tissue metastasis along with boosted assembly of ROS (hydrogen peroxide and superoxide) and rises protein oxidation, lipid peroxidation, interrupts enzymes and Deoxyribonucleic acid (DNA), impairs cell proliferation, and endorses apoptosis (Mannan et al., 2021).

1.2.2. Metabolism of arsenic

Sodium arsenate has been extensively utilized to evaluate arsenic exposure because of the main pathways (oxidative methylation and glutathione conjugation) involved in arsenic metabolism. Inorganic arsenic by entering into blood vessels from the digestive organs is methylated in the liver into dimethylarsonic acid (DMA) and monomethylarsonic acid (MMA) forms (Castriota et al., 2020).This is the pathway through which inorganic arsenic is transformed to MMA and DMA by interchanging the pentavalent arsenic valency

change into trivalent arsenic. This inorganic arsenic along with its metabolites by crossing the BBB collect in the brain tissue (Khairul et al., 2017).

1.3. Arsenic induced impairment in memory and learning

The hippocampus is vital to functions of spatial navigation and memory formation. Hippocampal lacerations harvest devastating declarative memory impairment in nonhuman primates and in humans (Lisman et al., 2018). There is established evidence about hippocampal neurons role in the development of memory and capacity for learning and memory is decreased by impairment of hippocampal neurons due to arsenic toxicity (Pandey et al., 2017). Switching attention and pattern memory and intellectual dysfunction in children have been caused after chronic sodium arsenate exposure. Changes in learning task and neurobehavioral alterations in rats as a result of sodium arsenite exposure have been evaluated in a study conducted by Dominguez et al, (2021).

Compromised cognitive and motor function has been presented in adult animals exposed to arsenic for both long and short time period (Karim et al., 2019). Diminished memory power, decrease in attention, and negotiated learning abilities after arsenic exposure in humans have been investigated (Tyler and Allan, 2014). Rats exposure to arsenic contaminated water for long period decreased the expression level of NR2A mRNA in hippocampus along with compromised ability of learning in rats (Luo et al., 2012).

1.4. Synaptic Plasticity

Synaptic plasticity is taken into consideration as the mechanism of neuronal cells for memory, and it refers to change in the strength of synaptic transmission or synapses between two neurons which is often supplemented by physical change of the synapses. There are different forms of synaptic plasticity displayed by different brain regions including memory consolidation which is potential of excitatory synapsis to maintain long term transmission also defined as an ongoing reinforcement dependent on experience in the efficiency of transmission at synapsis (Cornell et al., 2022).

1.4.1. Impairment of hippocampal synaptic plasticity post-exposure to arsenic

Exposure to arsenic in mouse leads to decreasing synaptic plasticity (Sheng et al., 2021); cell specification and neurogenesis in the hippocampus (Tyler and Allan, 2013). Arsenic exposure reduced synaptic proteins expression in murine pups (Amal et al., 2020; Dixit et al., 2020), diminished barbarization, granule cells and pyramidal neurons with condensed number of spines (Nino et al., 2021; Zhao et al., 2017). High absorption of arsenic in the brain upsets signaling pathways and functional association of neurons with other cells causing neurological dysfunction and persuaded synaptic transmission (Lu et al., 2014).

Decrease in hippocampal long-term potentiation and synaptic transmission has been reported in rodents with exposure to high arsenic concentration during initial phases of growth and adulthood (Nelson-Mora et al., 2018) being secondary to rehabilitated glutamate transport (Wang et al., 2018). In vitro exposure to arsenite metabolites has been

established to cause parallel alterations in plasticity and synaptic transmission in hippocampal slices (Nino et al., 2022). Another study reported compromised expression of Synaptophysin (SYN) and Post synaptic density protein (PSD-95) in the hippocampus, cortex and cerebellum of offspring rats due to perinatal arsenic exposure showing its potential to decrease synaptic density and causing typical autism-like alteration in brain cells, which could subsidize behaviors related to autism in children (Zhang et al., 2022).

OBJECTIVES OF STUDY

- To investigate the effect of MeCbl on synaptic gene expression in the hippocampus of rats with arsenic induced neurotoxicity.
- To understand the effects of MeCbl on histopathological changes induced by arsenic.
- To examine the level of antioxidant enzymes after MeCbl supplementation in arsenic exposed rats.

LITERATURE REVIEW

2.1. Environmental arsenic exposure and associated pathologies in human

Environmental pollutions and the level of environmental toxic components has increased to alarming level due to expansion in number of factories and establishment of industries for the benefits of society (Ahmed et al., 2022). Arsenic is a metalloid element existing as elemental, organic, and inorganic forms as a natural compound of earth crust (Nurchi et al., 2020) and one of the environmental neurotoxicant with wide range of distribution in water, air and soil ranking first enlisted by the Agency for Toxic Substances and Disease Registry (ATSDR) being most harmful environmental compound (Khan et al., 2022).

According to World Health Organization (WHO), estimated population exposed to inorganic arsenic contaminated water with a concentration limit exceeding over 10µg/L, is more than 200 million people globally (Castriota et al., 2020). This high level arsenic exposure in developing countries is crossing the maximum promising concentration (Ramsay et al., 2021). More than 50% of groundwater wells in Pakistan contain increased concentration of arsenic in drinking water and based on the available data, it is predicted that almost 47 million population is suffering with various ailments due to the arsenic-contaminated water (Sharma et al., 2018). Due to unavailability of data from newly affected areas, this number is expected to increase (Li et al., 2021). Sources of arsenic contamination and resulting health effects are given in Table 2.1.

Table 2.1 Exposure of arsenic and associated pathologies in human

Sources of exposure/entry	Pathologies in human	References
Intake of arsenic contaminated water, medicines and food	Diseases of physiological systems in accidental, environmental and occupational setting	(Crinnion, 2017)
Consumption of rice contaminated with arsenic	Skin pigmentation and lesions, pink eye infection, anemia, keratosis, neuropathic pain, respiratory diseases, different types of malignancies	(Rokonuzzaman, 2022; Singh et al., 2017)
Dust and smoke containing arsenic in steel industry	Cancer of body organs, noncancerous diseases of heart, nervous system, kidney and liver	(Rahaman et al., 2021; Ahmed et al., 2022)
Drinking water contamination with industrial, agricultural and natural wastes	Cardiovascular diseases, developmental abnormalities, hearing loss, diabetes, lung and liver fibrosis, reproductive and neurological problems, cancer and black foot disease	(Kaur et al., 2022; Branca et al., 2018)
Anthropogenic and agricultural residues including wastes from smelting of metals, glass, semiconductor manufacturer and mining industries, production of fertilizers	Multiple cancers, neuronal, cardiovascular, metabolic and developmental disorders, learning problems, depressive behavior, neural tube and intelligence processes developmental abnormalities	(Rehman et al., 2022; Baker et al., 2018)
Intake of contaminated drinking water, food, dermal absorption and inhalation of contaminated air	Impairment in cognitive functions, memory and learning functions alterations during childhood, polyneuropathy, nervous system disorders including neurodegeneration, poor concentration, peripheral neuropathy, Parkinson's disease, verbal comprehension problems, Guillain–Barre-like neuropathy, encephalopathy	(Castriota et al., 2020; Escudero-Lourdes., 2016)

2.1.1. Mechanism of action of arsenic induced neurotoxicity

Disintegration of tissues due to increased production of ROS leading to alterations in the morphology of chromosomes, cross linking of protein and damage to genetic message of the cells due to production of oxidative biomarkers (8-Hydroxy-2′-deoxyguanosine), human cells with damaged genetic information and disruption of cytoskeletal structure is characterized as arsenic cytotoxicity. The proposed mechanism for neurotoxicity caused by arsenic majorly comprises OS along with increased lipid peroxides, DNA damage and ROS besides reduction in glutathione levels and altered metabolic rate of several neurotransmitters (acetylcholine, glutamate, gamma amino butyric acid and monoamines) and disorganization of cytoskeletal structure with mitochondrial dysfunction leading to axonal degradation and reduced nerve conduction velocity having role in peripheral neuropathy. Neuronal apoptosis resulting in Parkinson's disease has been studied due to increased OS. Also, downregulation of many synaptic plasticity markers has been reported to alter the synaptic transmission leading to impairment in learning and memory (Figure 1.2). Although, pathophysiological mechanisms involved in arsenic-induced neurotoxicity have been deeply studied but still neurotoxicity due to arsenic is inconsistent in the animal models developed for arsenic neurotoxicity because of variation in duration, doses and different salts used (Branca et al., 2018). Some of the methods for the development of the animal model for arsenic-induced neurotoxicity are listed in (Table 2.4).

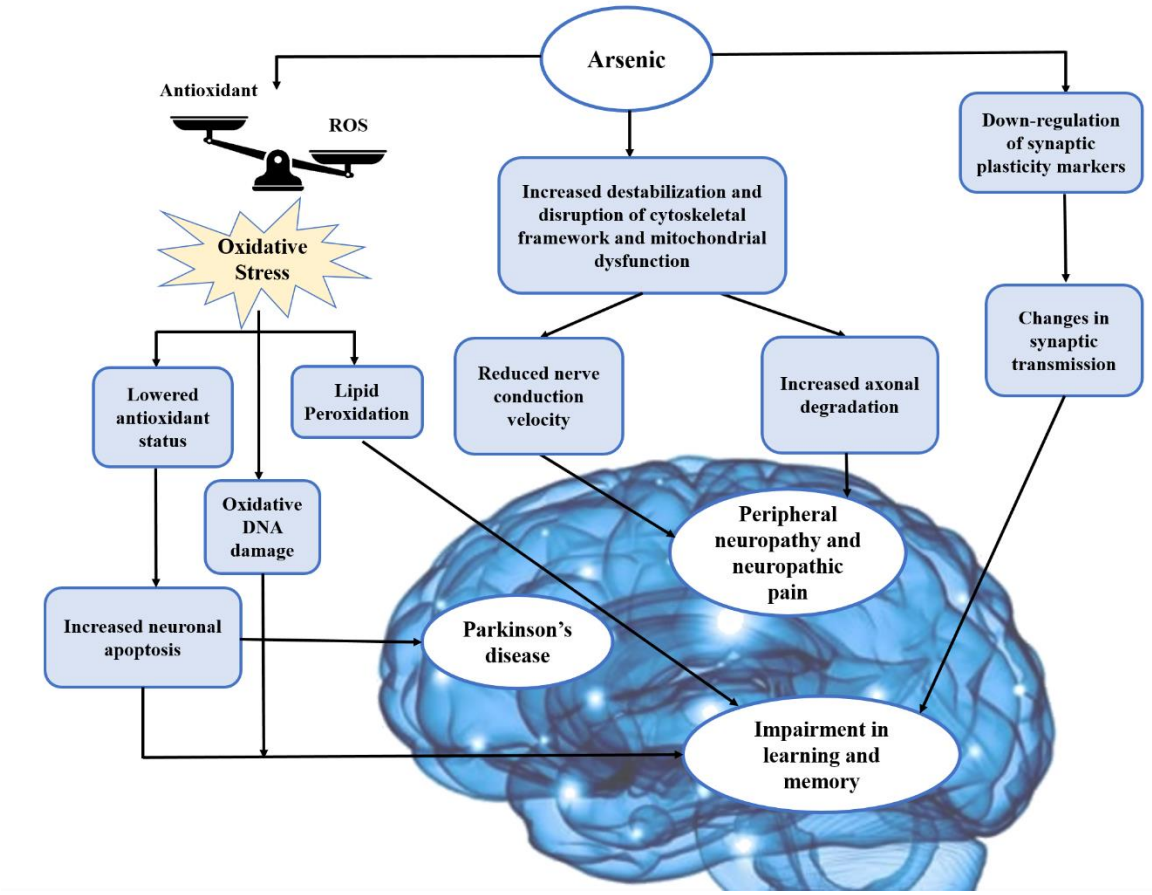


Figure 2.1. Mechanism of action of neurotoxicity caused by arsenic. OS; oxidative stress, ROS reactive oxygen species (Modified from (Branca et al., 2018)).

Synaptic transmission alteration, autophagy, oxidative stress, neurotransmitter homeostasis, cellular apoptosis and epigenetic modification alteration have been anticipated as a mechanisms for arsenic neurotoxicity (Watanabe and Hirano, 2013; Luo et al., 2012). Commonly accepted theories concerning the mechanism of arsenic neurotoxicity include cell apoptosis, provoking oxidative DNA damage, excessive oxidative stress, pathological changes and mitochondrial dysfunction (Escudero-Lourdes, 2016; Pachauri et al., 2013; Yen et al., 2012). Free radicals attack the nervous tissue of rat resulting in unusual death of neural cells post arsenic exposure and cause brain toxicity including changed cholinergic and monoaminergic signaling and behavioral deficits

including learning disability, memory impairment, and locomotory imbalance (Tyler and Allan, 2014).

2.2. Role of synaptic plasticity in memory and learning

Synaptic plasticity is vital for brain capability to modify and adjust according to novel information. There are several indications about the synaptic plasticity in the cells at molecular levels as the root of memory and learning (Stuchlik, 2014). Learning and memory are signified by hugely interrelated circuits of neurons with facilitated synapses that contribute a neuron in passage of signal (chemical or electrical) to the adjacent neuron. The synapse effectiveness is reinforced or deteriorated over time, and this is what called synaptic plasticity. As a result, synaptic plasticity is proposed as a vital substrate for memory and learning in the cells. The phenomenon of synaptic plasticity has been certainly acknowledged in the brain areas (cerebellum, hippocampus, striatum, cerebral cortex, and amygdala) associated with learning and memory (Tonegawa et al., 2018). Synaptic plasticity defined as a structural and functional modification of synapses, is found closely connected with memory and learning including the changes in the morphology of the synapses (Lüscher and Malenka, 2012; Magee & Grienberger, 2020). The physiology (synaptic plasticity and synaptic morphology) of learning and memory occurs in the hippocampus and any damage acts as a root of deficits in learning and memory (Zhang et al., 2021).

Table 2.2: Synaptic plasticity genes and their functions in learning and memory

Gene	Functions	References
Postsynaptic density-95 (PSD-95): Gene coding for protein at the excitatory synapses Post-synaptic density (PSD)	Glutamatergic transmission, synaptic plasticity, neurodevelopmental morphogenesis of dendritic spines	(Sandestiget al., 2020; Li et al., 2022; Cardoso et al., 2019)
Synaptophysin (SYN): Gene for pre-synaptic relative protein, CNS synaptic plasticity marker	Neurotransmitters release, formation of synapses, brain reliable synaptic plasticity marker	(Liu et al.,2013; Tönnies and Trushina et al., 2017; Davis et al., 2017)
Kalirin: Kalirin protein coding gene in the diffuse B lymphoma gene family in PSD along with PSD-95	Maturation and development of excitatory synapses	(Ma et al., 2014; Qiao et al., 2014; Duman et al., 2015)
Synaptosomal-associated protein (SNAP-25) Gene involved in synaptic transmission	High expression in CNS and role in synaptic plasticity, synaptic vesicle recycling and release, neurite extension, neuron repair, axonal growth, synaptogenesis, dendrite formation, synaptic vesicles fusion with membrane	(Daraio et al., 2018; Wang et al., 2018)
Homer: Genes for scaffolding homer 1–3 evolutionally conserved PSD key components linked with ion channels, receptors, and other scaffolding proteins	CNS intracellular calcium mobilization and glutamatergic signaling	(Chen et al., 2012; Suh et al.,2018; Haas and Strittmatter, 2016)
Calcium/calmodulin-dependent protein kinase IV (CAMK-IV): Serine/threonine protein kinase family post synaptic multi-functional enzyme coding gene	Synaptic plasticity, apoptosis, memory formation and consolidation, cell proliferation, neuronal growth, brain development, hematopoietic stem cell maintenance, T lymphocytes activation, inflammatory response	(Naz et al., 2016; Song et al., 2015)
SH3 And Multiple Ankyrin Repeat Domains (Shank 1 and 2): Genes coding for scaffolding proteins in the glutamatergic synapses PSD	Extensive protein complex organization at the excitatory glutamatergic synapses PSD	(Guilmatre et al., 2014; Jiang and Ehlers, 2013)
Neuregulins (Nlgn 1 & Nlgn 2): Post-synaptic transmembrane proteins at the excitatory synapses coding gene	Nlgn 1 supports excitatory specializations development based on alternative splicing, Nlgn 2 in inhibitory synapses specially stimulates inhibitory contacts formation	(Gan and Südhof, 2020)
Neurexin (Nrxn 1 and Nrxn 2): Brain-specific polymorphic proteins at PSD coded by 3 neurexin genes Nrxn 1, Nrxn 2 and Nrxn 3	Effect on synaptic remodeling	(Sindi et al., 2014)

Table 2.3. Synaptic plasticity gene expression alterations due to neurotoxic agent

Gene	Disease model	Alterations in expression	References
PSD-95	Prenatal bisphenol exposure in mice	Reduced expression of PSD-95, spatial and aversive memory impairment, anxiety-related behavior	(Wang et al., 2021)
	Methylmercury exposure in mice pups	Decreased expression of syntaxin and PSD -95 with reduction in neurons branching and dendritic length	(Wang et al., 2022)
	Sevoflourane exposure in neonatal rats	Reduced hippocampal synapse density, fear memory, radically decreases synaptic genes (kalirin and PSD-95) expression in the developing brain hippocampus	(Li et al., 2022)
SYN	Nitrite exposure in mice	Decreased number of visual cortex syn positive buttons	(Chen et al., 2019)
	Trimethyltin exposure in mice	Reduction in the hippocampal Syn protein level	(Zhang et al.,2014)
Kalirin	Sevoflourane exposure in neonatal rats	Reduced hippocampal synapse density, fear memory, radically decreased kalirin-7 and PSD-95 expression in the hippocampus during development	(Li et al., 2022)
SNAP-25	3% sevoflurane exposure in neonatal rats for 6h	Elevated expression of syntaxin along with SNAP-25 in the rat hippocampus	(Xiao et al., 2016)
	Acrylamide exposure in rats	Downregulation of SNAP-25 in cortex and hippocampus along with neuronal necrosis	(Fang et al., 2022)
	Arsenic exposure in balb/c mice	SNAP-25 and PSD-95 expression reduction leading to impaired memory and learning	(Ansari et al., 2022)
Homer	Lead (Pb) exposure in offspring rats	Reduced expression (SNX6) and respective decreased expression of homer 1 causing learning and memory deficiency	(Pang et al., 2019)
	Homer1 knockout mice	Overexpression of hippocampal Homer1c rescued memory and learning ability	(Gimse et al., 2018)

Gene	Disease model	Alterations in expression	References
CAMK-IV	Knockout mice	Disrupted hippocampal synaptic plasticity Long term impairment of memory	(Song et al., 2015)
	Overexpression of CAMK-IV in the forebrain of mice	Promotes fear long-term and social memory formation	(Jang et al., 2020)
	Arsenic-induced decrease in CaMK-IV expression in mice	Cerebellar long term depression leading to impaired spatial memory task performance	(Ghosh and Giese, 2015; Guan et al., 2016)
Shank 1 and 2	Bio metal profile for autism spectrum disorder in ex-vivo setting	Reduced synapse density due to lower level of Shank genes	(Hagmeyer et al., 2015)
	Lanthanum exposure in offspring rats	Significant reduction in expressions of both mRNA and proteins including SYN, PSD-95, Shank 3, synapsin and Shank 1 in the hippocampus with reduction in memory and learning ability	(Xiao et al., 2020)
Nlgn 1 and Nlgn 2	Lead exposure in rats	Down-regulating Nlgn 1 protein levels with altered spine plasticity in the developing hippocampus	(Zhao et al., 2018)
	Methamphetamine exposure in rats	Increased xpression of PSD-95, Nlgn 1 and synapsin 1	(Cao et al., 2022)
Nrxn 1 and Nrxn 2	6-hydroxydopamine leisons in rats	Reduced levels of PSD-95, Nlgn 1 and Nrxn1 in the hippocampus	(Sheibani et al., 2022)
	Bisphenol-A exposure in rodents	Impaired spatial memory through upregulating expression of Nrxn1 along with abnormal dendritic spine density in brain (cerebral cortex and hippocampus)	(Wang et al., 2021; Kumar and Thakur, 2014)

2.3. Therapeutic Strategies for arsenic induced neurotoxicity

Even though the precise mechanism and pathology of arsenic mediated neurotoxicity is not well established and still the researchers found no effective management but studies has reported that curcumin considerably reduces cholinergic dysfunction in brain induced by arsenic and also shows neuroprotective efficiency against neurotoxicity of arsenic (Yadav et al., 2021). Protocatechuic acid has neuroprotective effects on toxicity induced by sodium arsenate in mice (Li et al., 2021). Melatonin has been approved effective against oxidative stress due to arsenic and inhibited apoptosis, inflammatory chemicals and DNA damage and it is proposed that melatonin could be a favorable and important therapy for the effective management of arsenicism in medical circumstances (Abdollahzade and Majidinia, 2021).

2.4. Cobalamin and neurotoxicity

Vitamin B_{12} (Cobalamin- Cbl) is one of the essential nutrients (not synthesized in humans) and many health problems worldwide (anemia and neuronal dysfunction) are associated with its deficiency. Cbl is a dynamic force helping the body to make blood cells and DNA and it is critical to withstand a healthy nervous system particularly the brain (Green et al., 2017). Cbl is a necessary water-soluble vitamin vitally important in nervous system functions, hematopoiesis and maintenance of complete gastrointestinal mucosa and regulation of many other Cbl-dependent metabolic processes. The basic structure of Cbl consists of central cobalt atom surrounded with four reduced pyrrole rings called the corrin ring system. The vitamin is naturally present in foodstuff obtained from animals, such as milk, fish, meat and the suggested amount in humans can only be obtained by consuming balanced omnivorous diet (2.4 µg Cbl/day) (Green et al., 2017). Cbl retains anti-inflammatory and pain-relieving

properties (Hosseinzadeh et al., 2012) and it is reported to mitigate neuronal disintegration. It plays very important role in nucleotide synthesis because it is the source of a coenzyme contributing in folate metabolism (Chan et al., 2018). As a result, Cbl insufficiency can lead to neuropathy, serious blood deficiency and compromised brain function. Animal and human studies have well established the role of Cbl deficiency in peripheral neuropathy (Mehrdad et al., 2021). Cbl is recognized to have essential roles in the brain function throughout the life of an animal and also in the inhibition of ailments of CNS during development, mood disorders and dementias including vascular dementia and Alzheimer's disease (AD) in aged people (Lauer et al., 2022).

2.4.1. Cbl as an antioxidant and anti-inflammatory agent

Different mechanisms are involved for the accomplishment of antioxidant potential of Cbl including uninterrupted removal of ROS, principally superoxide in the mitochondria and cytosol (Chan et al., 2018) and secondarily exciting ROS scavenging by maintenance of glutathione (Karamshetty et al., 2018). Hippocampus cells, accountable for memory and learning are upset in AD, causing the production of numerous inflammatory markers (cyclooxygenase–2 (COX–2), Nrxn 1 –Nlgn, and caspase–3 proteins) used in measurement of AD development and severity. Cbl having role in brain functioning possess anti–inflammatory properties and neuron cell death in AD is associated with its impairment. Cbl has been reported to significantly improve molecular equilibrium in scopolamine–induced AD in rats and restored the synaptic plasticity. PSD–95 and Nrxn 1 and Nlgn concentrations were considerably reduced, whereas activated caspase–3 and COX–2 was boosted in the hippocampus of scopolamine–inoculated rats. Cbl administration decreased these alterations

and repressed hippocampal apoptosis and inflammation with the preservation of proteins (pre and post-synaptic) and probably hippocampal synaptic integrity (Mehrdad et al., 2021). In addition, clinical studies consistently displayed that Cbl alone or along with vitamin B family additional representatives have positive effects on brain atrophy, cognitive function and inflammation and in aged people without cognitive degeneration or in slight cognitive loss patients. AD patients were found to have reduced plasma level of Cbl in comparison of healthy control while B vitamins supplementation improved cognitive functions in several (randomized) clinical trials and in in many reported studies (Lauer et al., 2022).

2.4.2. The therapeutic uses of Mecobalamin

Methylcobalamin (commonly known as methyl B_{12} or mecobalamin - MeCbl) is vitamin B_{12} analog effective for the treatment or prevention of the pathology arising from vitamin B_{12} defeciency. MeCbl actively functions outside the mitochondrion in the CNS and is important for mitosis. MeCbl is effective for the management of degenerative disorders, diabetic neuropathy, and amylotropic lateral sclerosis initial treatment and also used to treat nutrition-dependent diseases (rheumatoid arthritis, anemia and memory loss) and promote regeneration of injured nerves. It alleviates the neurotoxicity induced by glutamate and also exhibits pain-relieving effects. It antagonizes pain behavior in neuralgia, pain in lower back, diabetic neuropathy, and stimulates nerve passage. It assists the body to harvest carbohydrates and fats to obtain energy (Zhang et al., 2013). Cognitive impairment could be improved with MeCbl and estradiol benzoate supplementation because of their re-myelination and anti-inflammatory properties and combination therapy of estradiol benzoate and MeCbl can be useful in the management of multiple sclerosis-induced dysfunction and memory problems

through stimulation of the hippocampal cAMP-response element binding protein, protein kinase B and abrineurin proteins (Taherian et al., 2021). Supplementation with folic acid, choline, riboflavin and MeCbl could encourage neuroplasticity after stroke through growing abrineurin levels and improved levels of abrineurin may be attributed to the protein kinase A mechanism (Jadavji et al., 2019).

Since arsenic-induced pathogenesis is commonly described by increase production of ROS, changes in genes related with synaptic plasticity and neuronal apoptosis, keeping in view the antioxidant and neuroplasticity related potential of MeCbl, we used MeCbl mixed in diet for attenuation of negative changes in anti-oxidant system, apoptosis and synaptic plasticity of hippocampus. The dose decided was adjusted according to previous literature listed in (Table 2.5).

Table 2.4. Animal models for Arsenic induced neurotoxicity

Animal model	Age	Body weight (grams)	Compound	Dose	Duration	Route	References
Male wistar rats	8 weeks	120-150	Sodium arsenite	25 ppm	8 weeks	Oral gavage	(Soni et al.,2018)
Male Wistar albino rats	8 weeks	180–200	Sodium arsenite	10 mg/kg/day	3 weeks	Per os (p.o) in drinking water	(Goudarzi et al., 2018)
Adult female pregnant rats	Not specified	Not specified	Sodium arsenite	4 mg/kg	6 weeks	p.o in drinking water	(Chandravanshi et al., 2019)
Male wistar rats	Not specified	200 ± 25	Sodium arsenate	10 mg/kg	11 days	Oral gavage	(Firdaus et al., 2018)
Adult female wistar rats	8-10 weeks	180–220	Sodium arsenite	50 mg/kg	3 weeks	Drinking water	(Peruru and Dodoala, 2021)
Female Wistar rats	Not specified	200–250	Sodium arsenite	7.5 and 200 μM	3 weeks	Drinking water	(Abdollahzade and Majidinia, 2021)
Male Sprague Dawley rats	Not specified	180 ± 20	Arsenic trioxide	2 mg/kg	6 months	Oral gavage	(Du et al., 2022)
Adult albino female mice	4-6 weeks	25–30	Arsenic trioxide	4 mg/kg	45 days	p.o in drinking water	(Mehta et al., 2021)
Female Wistar rats	Not specified	180+20	Sodium arsenite	50 mg/kg	28 days	p.o in drinking water	(Yadav et al., 2021)

Table 2.5. Animal models used for therapeutic effects of Cbl

Animal model	Body weight (Grams)	Compound	Disease model	Dose	Duration	Route	References
Adult male Wistar rats	200–250	Cbl	AD	(0.5, 2, and 4) mg/kg	14 days	Intraperitoneal injection	(Mehrdad et al., 2021)
Adult male Wistar rats	280–320	MeCbl	Scopolamine induced memory impairment and orafacial pain	0.5 µl	1 day	Intra-hippocampal microinjectio	(Erfanparast et al., 2017)
Wild type (C57BL/6J mouse) TCblR/*CD320* knockout mouse	Not specified	Cbl	transcobalamin receptor/CD320 Knockout mouse	50 µg/ kg/diet	Not specified	Diet	(Arora et al., 2017)
Wildtype male mice	Not specified	Cbl	Ischemia	0.5mg/kg/diet	4 weeks	Diet	(Jadavji et al., 2019)

CHAPTER 3

MATERIALS AND METHODS

3.1. Chemicals and ragents

Nitro blue tetrazolium (NBT), hydrogen peroxide (H_2O_2) stock solution (35%) and dithiobisnitrobenzoic acid (DTNB) were acquired from the British Drug House (BDH, Dorset, UK). Hydroxylamine hydrochloride ($H_3NO{\cdot}HCl$) and sodium arsenate ($Na_2HAsO_4.7H_2O$) were acquired from Sigma Chemical Co. (St. Louis, USA). Trizol was purchased from Invitrogen (USA). Reverse transcriptase (RT), Deoxynucleotide triphosphate (dNTPs) and Taq polymerase were obtained from Fermentas (Thermo Scientific, USA). MeCbl tablets (Wockhardt) were acquired from local pharmacy and mixed in the normal feed of animals. Sodium chloride (cat # SO0225) and ethanol were obtained from Merck, USA.

3.2. Ethic statement

The Institution Review Board at Atta Ur Rahman School of Applied Biosciences, NUST approved this study (IRB# 135). Principles established by the Institute of Laboratory Animal Research, Division on Earth and Life Sciences (National Institute of Health, USA) were followed to conduct all the experiments.

3.3. Animals

The laboratory animal house (LAH) (Atta Ur Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST) housed the animals under regulated environment and provided standard feed, distilled water and 12-

hour light/dark cycle (Iqbal et al., 2016). The rat room had controlled temperature of 25 ± 2 ^{0}C. Animal regular food was made up of rough fat 30%, 4% basic fiber, 9% crude protein and 10% moistness.

3.3. Study Design

Sodium arsenate was administered to animals through drinking water for 60 days while MeCbl tablets were crushed and mixed into their normal diet and co-supplemented with sodium arsenate for 60 days.

Table 3.1. Strategy of experimental groups used in study

S#	Group name	Treatment	Duration	No. of animals
1	Control	Distilled water Normal feed	60 days	5
2	Arsenic treated	80 mg per kg of body weight sodium arsenate in drinking water	60 days	5
3	Arsenic + MeCbl (5 mg)	80 mg per kg of body weight sodium arsenate in drinking water MeCbl (5 mg/kg) in diet	60 days	5
4	Arsenic + MeCbl (10 mg)	80 mg per kg of body weight sodium arsenate in drinking water MeCbl (10 mg/kg) in diet	60 days	5

Control group was given normal feed and water. Arsenic-treated group was given water with sodium arsenate (80 mg/kg). Both MeCbl treated groups were given sodium arsenate (80 mg/kg) mixed in water. MeCbl low dose group was given MeCbl (5 mg/kg) and MeCbl high dose group was given MeCbl (10 mg/kg) added in chow diet. The whole treatment was of 60 days (Table 3.1). The dose of 80 mg/kg of sodium arsenate means that

for every kg weight of animal, the concentration of arsenic intake should be 80 mg/kg. The dose decided for arsenic induced neurotoxicity development was taken from previous literature given in (Table 2.4.). The dose decided for MeCbl mixed in diet per kg of weight was decided according to previous publication listed in (Table 2.5). Prior to the initiation of experiment, daily water intake was measured and rats were weighed in order to calculate dose. Figure 3.1 is showing timeline of the experiment.

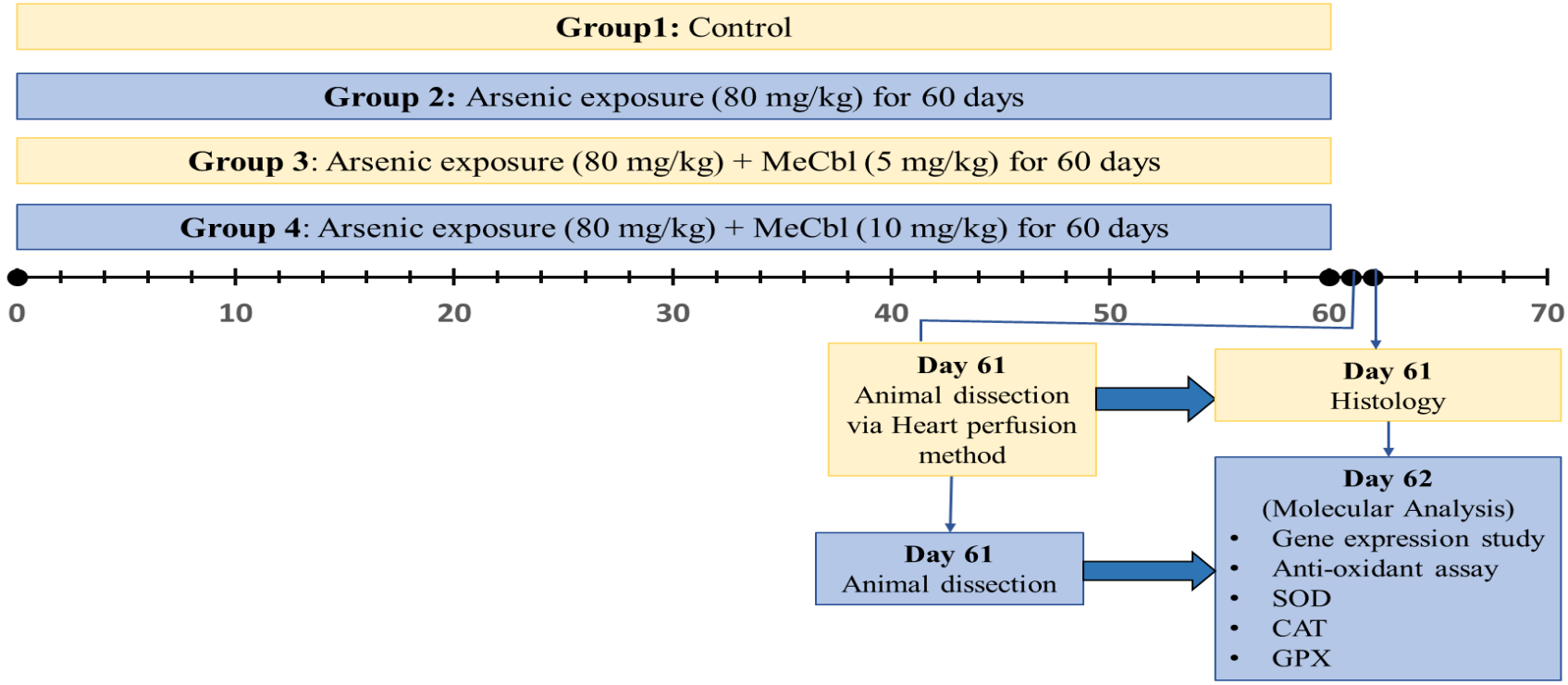


Figure 3.1: The timeline for the experiment

3.4. Body weights measurements

Body weights of animals were recorded twice a week for entire treatment duration (i.e., 2 months) for assessing the effect of arsenic and recovery with MeCbl treatment.

3.5. Histological Analysis of hippocampus

3.5.1. Tissue Perfusion/Fixation for assessing histological changes

The protocol followed for heart perfusion was according to Gage et al., 2012. Briefly, rats were weighed and anaesthetized using chloroform. A midline incision was made and sternotomy was done to expose the heart. A steady and gentle run of normal saline was permitted (5ml/minute) into left ventricle by injecting a needle to (5mm deep) while holding the heart with the forceps at stable position. A cut was made in the right atrium for the flow of blood. After injecting about 80 ml of normal saline, 100 ml of 4% paraformaldehyde solution was introduced through left ventricle and brain was excised and placed (4% paraformaldehyde) at 4˚C for 24 hrs before being processed further. Paraffin processing and entrenching was performed being in 4% paraformaldehyde after 24 hours, and the brain tissue was dehydrated through a sequence of alcohols (isopropanol), 70% (1 hr), 95% (1 hr), and 100% (1 hr) before paraffin infiltration. The brain tissues were then placed in xylene (4 hrs) and paraffin embedding was performed by keeping the tissue in molten paraffin (4 hrs at 60 °C) and then left to solidify (4 °C) in a mould (block formation) prior to cutting.

3.5.2. Haematoxylin and Eosin Staining

Standard haematoxylin–eosin (H and E) staining on 5µm tissue section (using SLE Mainz microtome CUT 6062) was performed. Tissue after being deparaffinized was incubated (8 minutes) in Mayer's haematoxylin solution and for 10 minutes, washed in warm water. 95% ethanol was used to dip the sections and counterstaining was done with eosin (30 s).

3.5.3. Quantification of cell number

Cell number was analyzed quantitatively in three areas of hippocampus (dentate gyrus (DG), Cornu ammonis 1 (CA1) and Cornu ammonis 3 (CA3). Areas of 10,000µm^2 were randomly selected from each region and cell number was calculated (Image J software). The average values of all three areas in the hippocampus were taken and plotted.

3.6. Brain Dissection & Hippocampus Isolation

The rat was anesthetized using chloroform. The head was gently but assertively strained forward and posterior to the ears, a cut was made with medical scissors. A slight incision was made starting from caudal point followed by making a safe cut through the anterior half exposing the skull. The forceps slanted and broken off the parital bone of both sides of the brain lifting the anterior part of the brain out of the skull by using curved narrow pattern forceps. The excised brain was directly moved to a petri dish with pre chilled Phosphate Buffer Saline (PBS) in such a way that the ventral half of the brain was facing the plate. Olfactory bulb and cerebellum were removed using scalpel after shifting the brain

in the petri dish. Small curved forceps was then held in a closed position between the cerebral halves. Opening of cortical halves was revealed by placing the forceps between the cerebral hemispheres in a closed position and gently lifting them up. After obtaining sufficient opening along the middle, an angle of 30-40° was maintained for closed position forceps by directing it counterclockwise and then clockwise to separate the and right and left hippocampus from the cortex respectively. The dichotomized hippocampus was shifted in a pre-chilled eppendorf and stored till further use at -80 °C.

3.7. Biochemical Test

Hippocampal homogenate (10% or 0.5 ml) was added in a mixture containing Na_2CO_3 (1 ml from 50 mM), 0.4 ml of NBT (24 μM) and EDTA (0.2 ml from 0.1 mM). To start the reaction, 0.4 ml of hydroxylamine hydrochloride (1 mM) was added at the end. A shift in absorbance was recorded at 560 nm (25 °C) twice (time zero and after 5 min). Hippocampal homogenate was not added in suitable control along each batch of samples. U/g of brain was used to represent SOD activity whose 1 unit is equal to the enzyme quantity for inhibiting NBT reduction by 50%. For CAT activity, hippocampal homogenate (10% or 0.1 ml) was added into the reaction mixture including phosphate buffer (1 ml of 0.01 M) with adjusted pH of 7.4 and H_2O_2 (1 ml of 0.2 M). Incubation of tubes was done at 37 C (90 s). Then, dichromate reagent (5%- 2 ml) was added to stop the reaction. Incubation of samples was done for 15 min (100°C) and absorbance was taken at 570 nm to conclude the consumption of H_2O_2. Without addition of H_2O_2, fitting control was also carried out along all samples. The activity of CAT was presented as how much H_2O_2 was consumed in terms of μmol/min/g of brain. For measuring GPx activity, 0.3 ml of

hippocampal supernatant (10%) was added to the reaction mixture containing phosphate buffer (0.1 M with adjusted pH 7.4), sodium azide (10 mM, 0.1 ml), reduced glutathione (2 mM, 0.2 ml) and H_2O_2 (1 mM, 0.1 ml). Then incubation of mixture was done for 15 min at 37 °C and by adding TCA (5%) reaction was stopped. Centrifugation of tubes was done for 5 min at 1500 × *g*, and in 0.1 ml of collected supernatant, 2 ml of phosphate buffer (0.1 M, pH 7.4) and 0.7 ml DTNB (0.4 mg/ml) were added. The mixture reading was taken at 420 nm. Activity of GPx was presented in terms of as μmol/min/g of brain (Haider et al., 2015).

3.8. Gene expression analysis

3.8.1. RNA extraction

Ribonucleic acid (RNA) extraction was done according to the protocol designed by manufacturer using Tri-reagent. Firstly, brain tissues were washed with phosphate buffer saline (1X PBS) and homogenized in 1 ml trizol using sonicator (UP400S Hielscher Ultrasound Technology) and then for nucleoprotein complex dissociation, the mixture was allowed to stand at 25 °C (5 min). After adding 200 µl of chloroform, samples were thoroughly shaken (15) sec and then left at room temperature for next 10 min. The mixture was centrifuged (HERMLE Labortechnik GmbR, Germany) at 12,000 revolutions per minute (rpm) at 4˚C for time (15 min). The upper (colourless and aqueous) phase was removed cautiously and transferred to a new tube after centrifugation. Isopropanol (500 µl) was added to each tube with sample and left for 10 min (room temperature) for incubation. Centrifugation of samples were done again at 12,000 rpm for 10 min (4˚C). The RNA was precipitated out after centrifugation and a pellet was formed on inner side of the tube. The

upper layer without pellets was removed and were given wash with 1 ml of ethanol (75%) per 1 ml of trizol used in sample preparation and ethanol was diluted in DEPC water to deactivate RNase enzyme. Later, samples were centrifuged at 7500 Relative centrifugal force (rcf) (5 min) at 4˚C. The final RNA samples were stored at -80˚C until further used.

3.8.2. Quality of RNA

Before re-suspending in PCR water (30 µl), the pellet was allowed to dry. 2% agarose gel was used to assess the quality of RNA (obligatory for reproducibility and precision of the results). RNA bands obtained were then visualized on Wealtech Dolphen Doc (S/N470883) Gel Documentation system. The ratio of 28S to 18S rRNA on the agarose gel showed the quality of RNA which was comparable among all samples.

3.8.3 Real Time - Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for cDNA Synthesis

RNA (3 µg in 25 µl reaction volume) for RT-PCR was used to make cDNA. The protocol used for RT-PCR reaction included 2µl of 10mM dNTP's, 1µl of 5mM Oligo dT (heated for 5 min at 55°C) and 4µl of 5x RT buffer. 24µl reaction volume was made by adding PCR water accordingly and revert aid (1µl) is added at last.

3.8.4. Quantitative Real Time Polymerase Chain Reaction (qPCR) to analyze gene expression

Real time PCR was performed in ABI Prism 7300 Sequence Detection System (Applied Bio system, 7300). The PCR reaction mixture consisted of 13µl Master Mix (SYBR Green), 1µl of each forward and reverse primers, specific for particular genes,1µl of cDNA template and the volume was adjusted to 20 µl by adding DNase water. The thermocycling conditions were 50 °C for 2 m, 95 °C for 10 m followed by 40 cycles of 30 s at 95 °C, 1 m at 60 °C followed by 1 m at 72°C, and a final dissociation step. Dissociation curves, amplification plots and quality of the PCR products was verified by agarose gel electrophoresis. Each sample was tested in duplicate. β-actin values were used for normalization of other gene values. Analysis of the obtained values from three independent experiments was done relative to gene expression data using the 2-ΔΔCT method (Rao et al., 2013). The specific primer sequences of Syn, Kalirin, PSD-95, homer, SNAP-25, CAMK-IV, Nrxn 1 & Nrxn 2, Shank 1 & Shank 2, Nlgn 1 & Nlgn 2 are listed in (Table 3.2).

Table3.2. Primer Sequences of each studied gene along with conditions

Gene Symbol	Primer sequence	Annealing Temp
SYN	F= CATTCAGGCTGCACCAAGTG R= TGGTAGTGCCCCCTTTAACG	60°C
SNAP-25	F= CATCAGTGGTGGCTTCATCC R= CATGATCCTGTCAATCTGGCG	62°C
Nrxn 1	F= CTGATGATGGGCGACCAAGG R= TCAGCCGATTTCCCTGTGTG	64°C
Nrxn 2	F= CAACCTGTCCCTCAAGTCCG R= ACCATAGCGTGTCCAATCCC	64°C
PSD-95	F= TACGACAAGACCAAGGACTGC R= GGAATGAAGCCAATGTCGTCG	62°C
CAMK-IV	F= CGAAGATGCTCAAAGTCACGC R= ACTCCACCTCGAAGAAATCGC	62°C
Homer	F= TGACACAGAACTCAGAGCCAA R= TTGAGCGTGGCTAGTTCAGC	60°C
Kalirin	F= CGTGACGAATTTGGCCAGC R= CTGCCAGAAGTTGTCAGGCT	62°C
Nlgn 1	F= GCCCTTGGACCAAACTTGTCG R=TCTTTCAATCTGGGAGGCAGTG	64°C
Nlgn 2	F= GGCAGAAAGAACTCACGGCT R= GGCTGTCTCATTGCACTTCC	62°C
Shank 1	F= ACTATGGACTGTTCCAGCCAG R= CCGTCTTCGTGTGCAGTTTG	62°C
Shank 2	F=CGGAAGAGATAGTCCCAGCC R= GACCGTGGGCGTCATTACA	64°C
Actin β	F= ACGGTCAGGTCATCACTATC R= TTGGCATAGAGGTCTTTACGG	60°C

3.9. Statistical analysis

Graph Pad Prism software (Version 8.0.1) was used for statistical analysis. The statistical tests (One-Way and Two-Way Anova) applied to analyze the data followed by Bonferroni multiple comparison test. The significant P value was noted to be less than 0.05. Mean ± standard error of mean (SEM) was used to show the data.

CHAPTER 4

RESULTS

4.1. Arsenic effects and its treatment with MeCbl on body weights

Body weights of animals were recorded twice a week from treatment day 1 till the completion of the treatment (2 months). Arsenic-treated group (210.2 ± 2.47) exhibited substantial decrease ($p < 0.001$) in their body weights relative to all other groups from first till last week of the treatment. Body weights of Arsenic + MeCbl-treated group (5 mg) (227.5 ± 3.59) increased but it was also significantly different from control group (235.7 ± 4.31, $p < 0.05$). Arsenic + MeCbl-treated group (10 mg) (228.7 ± 5.41) showed body weight almost equal to that of control group (235.7 ± 4.31, $p > 0.05$) from first till last week of the treatment (Figure 4.1).

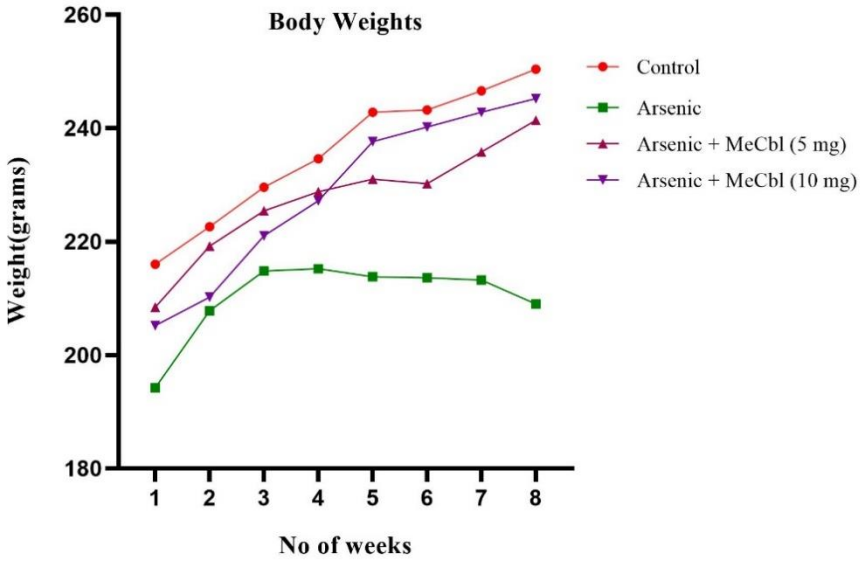


Figure 4.1: Arsenic effects and its treatment with MeCbl on Body Weights: Comparison of body weights for control group, arsenic, Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups. Mean ± SEM used to represent error bars using Graph pad prism (Version 8.0.1) for Two way ANOVA followed by Bonferroni's multiple comparison test.

4.2. Gene expression analysis of synaptic markers post arsenic and MeCbl exposure

4.2.1. mRNA expression of Post synaptic density-9

The level of PSD-95 expression in the hippocampus was significantly less arsenic-treated group and Arsenic + MeCbl-treated (5 mg) groups (0.18 ± 0.002, 0.34 ± 0.02 respectively) relative to control group (0.85 ± 0.02, $p<0.001$) but there was significantly higher expression of PSD-95 in Arsenic + MeCbl-treated group (5 mg) (0.34 ± 0.02) contrasted with arsenic-treated group rats (0.18 ± 0.002, $p < 0.01$). Arsenic + MeCbl-treated group (10 mg) (0.71 ± 0.03) also showed significantly lower expression of PSD-95 relative to control group (0.85 ± 0.02, $p < 0.01$) but notably higher expression level of PSD-95 in contrast with arsenic-treated and Arsenic + MeCbl-treated (5 mg) groups (0.18 ± 0.002, 0.34 ± 0.02, $p < 0.001$) (Figure 4.2).

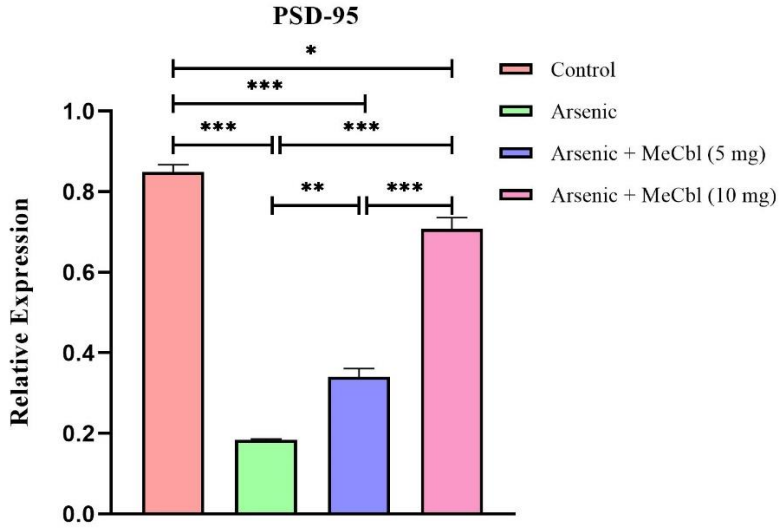


Figure 4.2: Transcriptional expression of hippocampal PSD-95. Histogram representing PSD-95 level for control group, arsenic, Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups (n=3). Mean ± SEM represented error bars using Graph pad Prism (Version 8.0.1) for One-way ANOVA, followed by Bonferroni's multiple comparison test. *=$p < 0.05$, **= $p < 0.01$ and ***=$p < 0.001$. For consistency, duplicates of each sample were run.

4.2.2. mRNA expression of Synaptophysin

The expression of SYN was significantly reduced as compared to control group (0.24 ± 0.006, $p < 0.001$) in hippocampus of both arsenic-treated and Arsenic + MeCbl-treated (5 mg) groups (0.14 ± 0.007, 0.14 ± 0.003). Expression remained same in both arsenic-treated and Arsenic + MeCbl-treated (5.mg) groups (0.14 ± 0.007, 0.14 ± 0.003, $p > 0.05$). The Syn expression was markedly lower in Arsenic + MeCbl-treated group (10 mg) (0.20 ± 0.006) in contrary to control (0.24 ± 0.006, $p < 0.01$) but it showed significantly increased expression when compared with both arsenic-treated and Arsenic + MeCbl-treated (5 mg) groups (0.14 ± 0.007, 0.14 ± 0.003, $p < 0.001$) (Figure 4.3 A).

4.2.3. mRNA expression of Kalirin

The expression of kalirin was lower significantly in all treated groups as opposed to control group (0.54 ± 0.02, $p < 0.001$) in hippocampus. Similar expression of kalirin was observed between arsenic-treated and Arsenic + MeCbl-treated (5 mg) groups (0.08 ± 0.005, 0.14 ± 0.007, $p > 0.05$). Arsenic-treated and Arsenic + MeCbl-treated (5 mg) groups (0.08 ± 0.005, 0.14 ± 0.007) showed significantly lower expression level of kalirin when compared to Arsenic + MeCbl-treated group (10 mg) (0.38 ± 0.02, $p < 0.001$) (Figure 4.3 B).

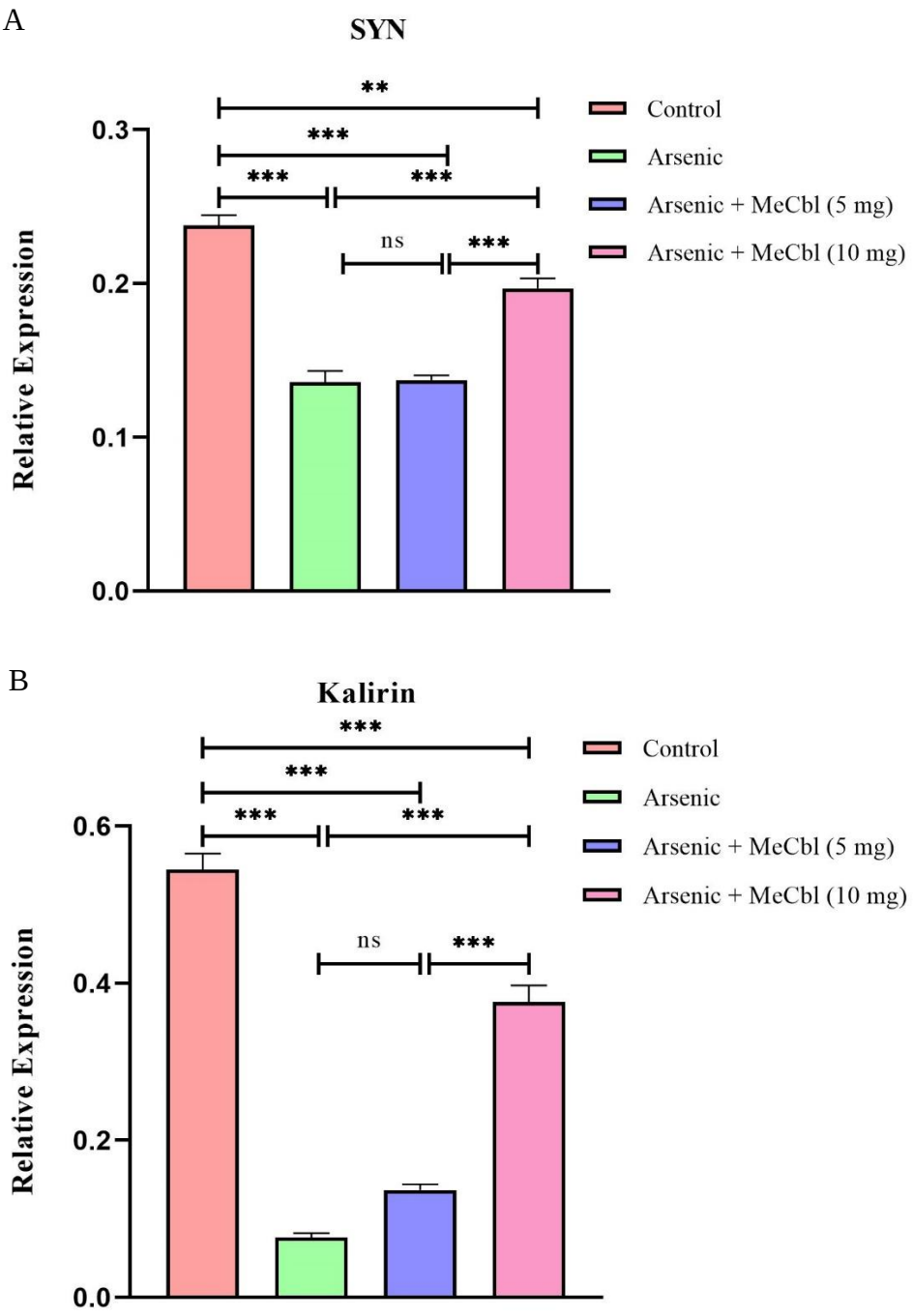


Figure 4.3: Transcriptional expression of hippocampal SYN and Kalirin. Histogram representing (A) SYN and (B) Kalirin level for control group, arsenic, Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups (n=3). Mean ± SEM represented error bars using Graph pad Prism (Version 8.0.1) for One-way ANOVA, followed by Bonferroni's multiple comparison test. n.s= $p > 0.05$, *=$p < 0.05$, **= $p < 0.01$ and ***=$p < 0.001$. For consistency, duplicates of each sample were run.

4.2.4. mRNA expression of Synaptosomal associated protein-25

Arsenic-treated and Arsenic + MeCbl-treated (5 mg) groups (0.27 ± 0.008, 0.27 ± 0.01) showed slight decline in the hippocampal expression of SNAP-25 relative to normal control (0.32 ± 0.008, $p < 0.05$). Arsenic + MeCbl-treated group (10 mg) (0.25 ± 0.003) showed more significantly lower expression of SNAP-25 in contrast with control group (0.32 ± 0.008, $p < 0.01$). While arsenic-treated group (0.27 ± 0.008) expression remained same as compared to Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups (0.27 ± 0.01, 0.25 ± 0.003, $p > 0.05$). Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups (0.27 ± 0.01, 0.25 ± 0.003, $p > 0.05$) showed similar expression (Figure 4.4 A).

4.2.5. mRNA expression of Calcium calmodulin dependent protein kinase-IV

There was substantial decrease in the expression of CAMK-IV in hippocampus in both arsenic-treated and Arsenic + MeCbl-treated (5 mg) groups (0.19 ± 0.005, 0.33 ± 0.02) as compared to control group (0.63 ± 0.04, $p < 0.001$) but Arsenic + MeCbl-treated group (5 mg) (0.33 ± 0.02) showed significant increase in expression when compared with arsenic-treated group (0.19 ± 0.005, $p < 0.05$). The expression of CAMK-IV was significantly lower in Arsenic + MeCbl-treated group (10 mg) (0.45 ± 0.02) relative to control group (0.63 ± 0.04, $p < 0.05$) but notably higher expression than that of arsenic-treated group (0.19 ± 0.005, $p < 0.001$). Expression remained same in both Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups (0.33 ± 0.02, 0.45 ± 0.02, $p > 0.05$) (Figure 4.4 B).

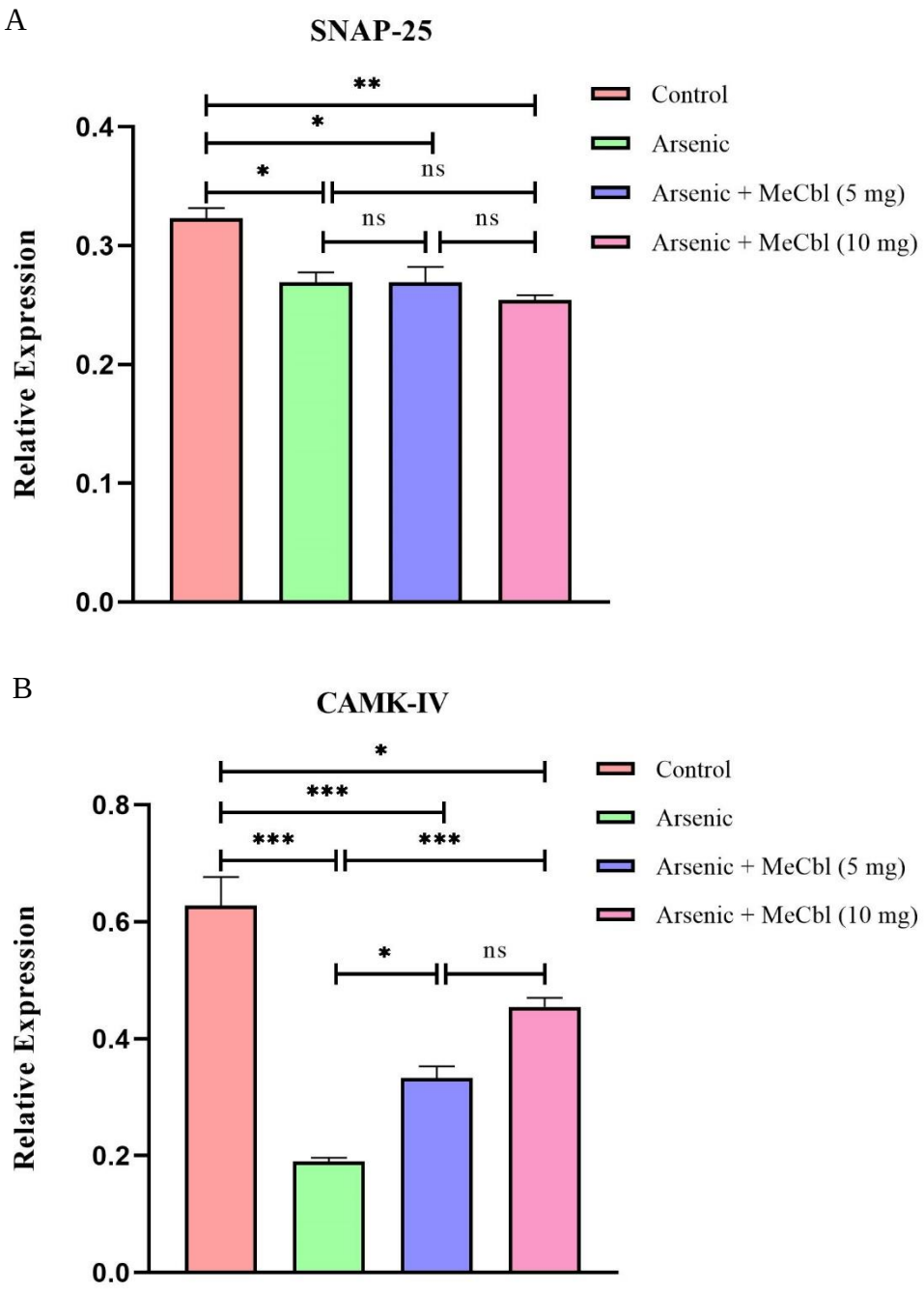


Figure 4.4: Transcriptional expression of hippocampal SNAP-25 and CAMK-IV. Histogram representing (A) SNAP-25 and (B) CAMK-IV level for control group, arsenic, Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups (n=3). Mean ± SEM represented error bars using Graph pad Prism (Version 8.0.1) for One-way ANOVA, followed by Bonferroni's multiple comparison test. n.s= $p > 0.05$, *=$p < 0.05$, **= $p < 0.01$ and ***=$p < 0.001$. For consistency, duplicates of each sample were run.

4.2.6. mRNA expression of Homer

All treated groups showed comparable expression level of homer in the hippocampus to the control group (0.46 ± 0.01, $p > 0.05$). There was no significant decrease in the expression level of homer in arsenic-treated, Arsenic + MeCbl-treated (5 mg) and Arsenic + MeCbl-treated (10 mg) groups (0.46 ± 0.005, 0.27 ± 0.01, 0.46 ± 0.01 relative to control group (0.46 ± 0.01, $p > 0.05$) (Figure 4.5).

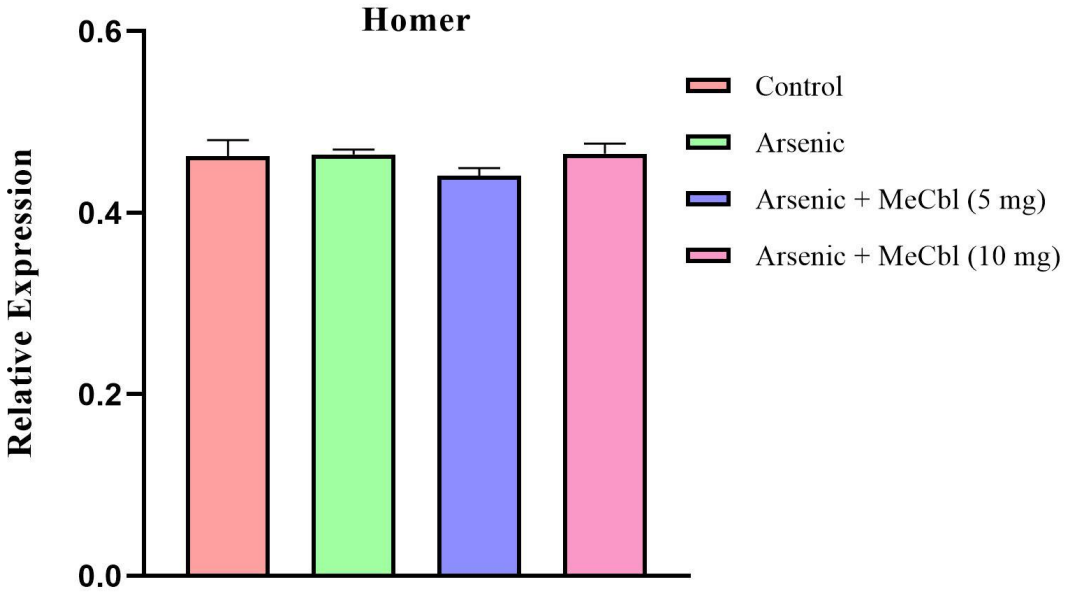


Figure 4.5: Transcriptional expression of hippocampal homer. Histogram representing homer level for control group, arsenic, Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups (n=3). Mean ± SEM represented error bars using Graph pad Prism (Version 8.0.1) for One-way ANOVA, followed by Bonferroni's multiple comparison test. n.s= $p > 0.05$. For consistency, duplicates of each sample were run.

4.2.7. mRNA expression of SH3 And Multiple Ankyrin Repeat Domains 1 and 2

Shank 1 expression level in hippocampus was considerably lower in arsenic-treated group (0.16 ± 0.003) by contrast with control group (0.24 ± 0.006, $p < 0.01$) but it showed no change in expression as compared to both Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups (0.20 ± 0.004, 0.17 ± 0.02, $p > 0.05$). There was also notably less expression in Arsenic + MeCbl-treated group (5 mg) (0.17 ± 0.02) relative to control group (0.24 ± 0.006, $p < 0.05$) but same expression was observed as compared to arsenic-treated and Arsenic + MeCbl-treated (10 mg) groups (0.16 ± 0.003, 0.20 ± 0.004, $p > 0.05$). Arsenic + MeCbl-treated group (10 mg) (0.20 ± 0.004) showed similar expression with that of control group (0.24 ± 0.006, $p > 0.05$) (Figure 4.6 A).

Arsenic-treated and Arsenic + MeCbl-treated (5 mg) groups (0.31 ± 0.003, 0.31 ± 0.01) showed significantly lower expression of shank 2 in hippocampus as compared to control group (0.46 ± 0.02, $p < 0.001$) but no significant difference was observed between both treated groups ($p > 0.05$). A significantly lower expression was observed in Arsenic + MeCbl-treated group (10 mg) (0.37 ± 0.006) relative to control group (0.46 ± 0.02, $p < 0.01$). Arsenic + MeCbl- treated group (10 mg) (0.37 ± 0.006) showed significant increase in the expression in contrast to arsenic-treated group (0.31 ± 0.003, $p < 0.05$) but same expression was observed in both Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups (0.31 ± 0.01, 0.37 ± 0.006, $p > 0.05$) (Figure 4.6 B).

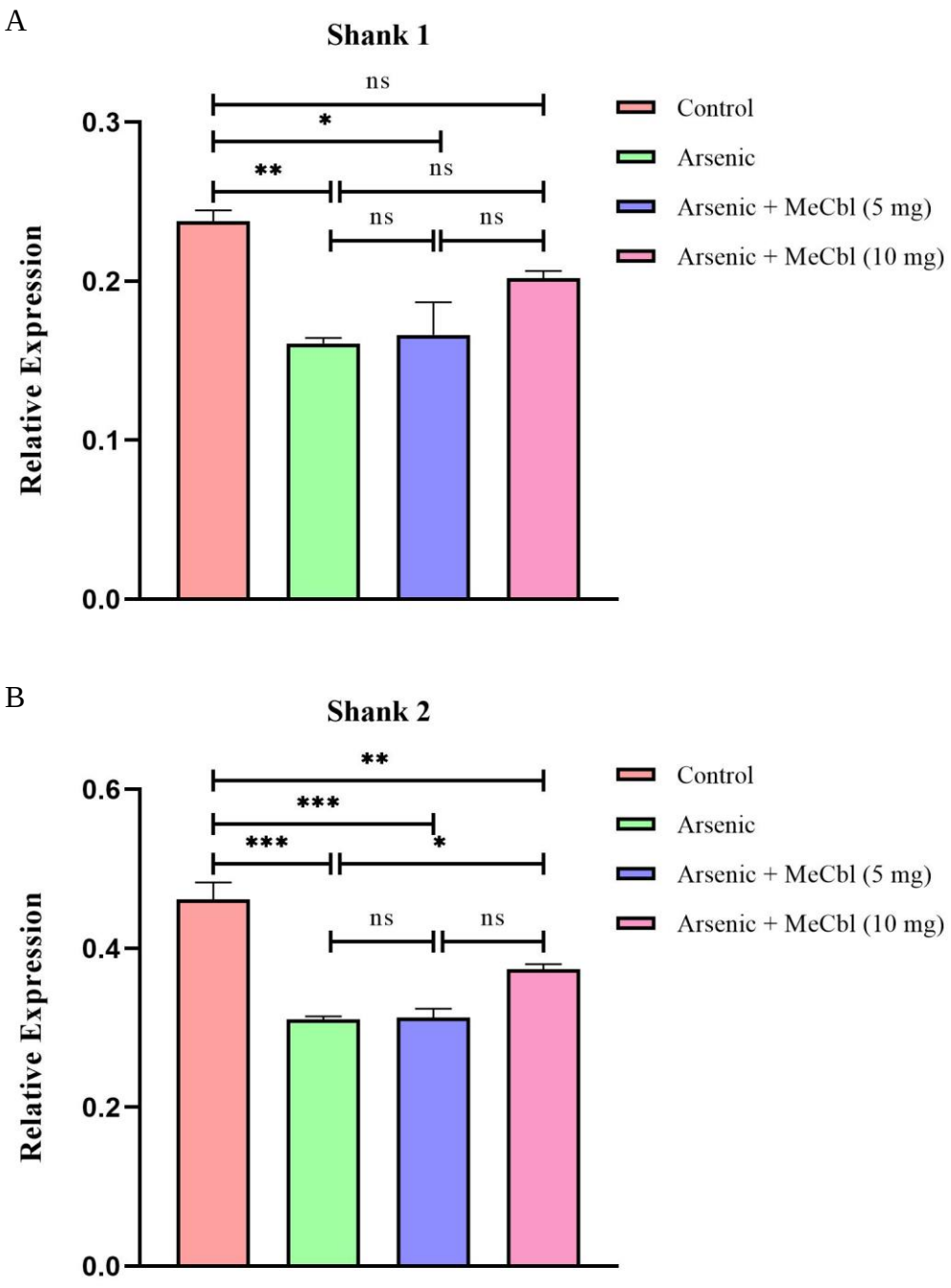


Figure 4.6: Transcriptional expression of hippocampal Shank 1 and Shank 2. Histogram representing (A) Shank 1 and (B) Shank 2 levels for control group, arsenic, Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups (n=3). Mean ± SEM represented error bars using Graph pad Prism (Version 8.0.1) for One-way ANOVA, followed by Bonferroni's multiple comparison test. n.s= $p > 0.05$, *=$p < 0.05$, **= $p < 0.01$ and ***=$p < 0.001$. For consistency, duplicates of each sample were run.

4.2.8. mRNA expression of Neregulin 1 and Neregulin 2

A remarkable lower expression of Nlgn 1 in the hippocampus was observed in arsenic-treated and Arsenic + MeCbl-treated (10 mg) groups (0.66 ± 0.01, 0.64 ± 0.01) as compared to normal control (0.42 ± 0.006, $p < 0.001$) and the expression was alike in both treated groups ($p > 0.05$). Arsenic + MeCbl-treated group (5 mg) (0.54 ± 0.01) showed significant reduced expression relative to control group (0.42 ± 0.006, $p<0.01$) but remarkably increased expression was observed when compared to arsenic-treated group (0.66 ± 0.01, $p < 0.01$). The expression was same in both Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups (0.54 ± 0.01, 0.64 ± 0.01, $p>0.05$) (Figure 4.7 A).

Arsenic-treated group (0.37 ± 0.01) showed notably reduced expression of hippocampal Nlgn 2 relative to control group (0.43 ± 0.01, $p < 0.05$) but it was alike relative to both Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups (0.35 ± 0.01, 0.36 ± 0.004, $p > 0.05$). Both Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups (0.35 ± 0.01, 0.36 ± 0.004) showed marked decrease in expression when compared to control group (0.43 ± 0.01, $p < 0.01$) and there was similar expression in two groups ($p > 0.05$) (Figure 4.7 B).

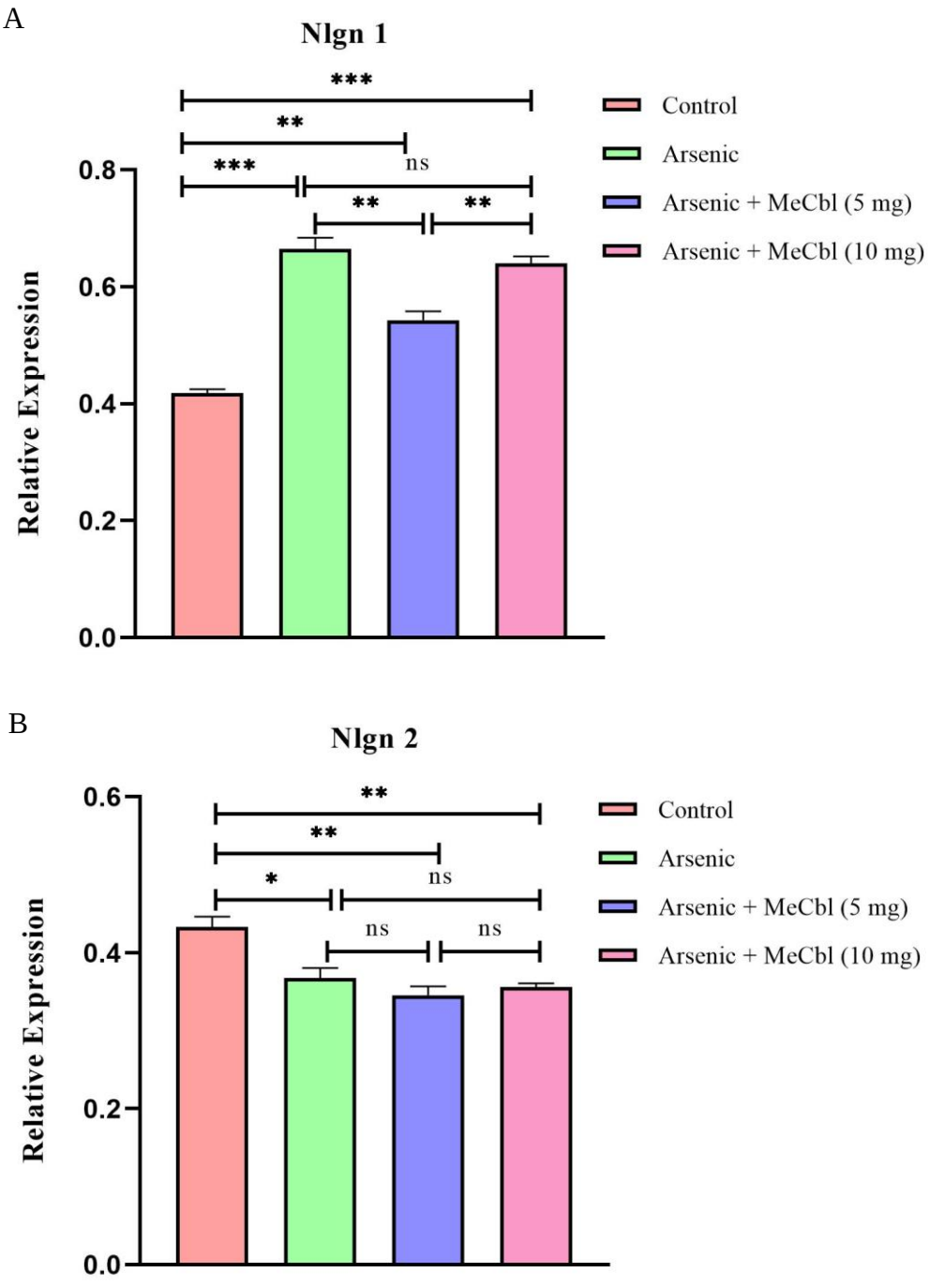


Figure 4.7: Transcriptional expression of hippocampal Nlgn 1 and Nlgn 2. Histogram representing (A) Nlgn 1 and (B) Nlgn 2 level for control group, arsenic, Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups (n=3). Mean ± SEM represented error bars using Graph pad Prism (Version 8.0.1) for One-way ANOVA, followed by Bonferroni's multiple comparison test. n.s= $p > 0.05$, *=$p < 0.05$, **= $p < 0.01$ and ***=$p < 0.001$. For consistency, duplicates of each sample were run.

4.2.9. mRNA expression of Neurexin 1 and Neurexin 2

Both arsenic-treated (0.18 ± 0.005) and Arsenic + MeCbl-treated (5 mg) (0.19 ± 0.006) groups showed marked decrease ($p < 0.01$) in the expression of Nrxn 1 relative to control group (0.26 ± 0.01) while no prominent difference was noted between the two groups and alike expression ($p > 0.05$) was observed between these treated groups. Arsenic + MeCbl-treated group (10 mg) showed notable decrease (0.20 ± 0.003, $p < 0.05$) in the Nrxn 1 expression level as opposed to control group (0.26 ± 0.01) but expression was alike ($p > 0.05$) when compared to both arsenic-treated (0.18 ± 0.005) and Arsenic + MeCbl-treated (5 mg) (0.19 ± 0.006) groups (Figure 4.8 A).

Expression of hippocampal Nrxn 2 was considerably lower in arsenic-treated group (0.24 ± 0.004) relative to control (0.34 ± 0.02, $p < 0.01$) but it showed similar expression relative to Arsenic + MeCbl-treated group (5 mg) (0.28 ± 0.005, $p > 0.05$). There was remarkabale decrease in expression in Arsenic + MeCbl-treated group (5 mg) (0.28 ± 0.005) in contrast to control group (0.34 ± 0.02, $p < 0.05$) and showed comparable expression with Arsenic + MeCbl-treated group (10 mg) (0.33 ± 0.004, $p > 0.05$). The expression was similar in both Arsenic + MeCbl- treated group (10 mg) and control group (0.33 ± 0.004, 0.34 ± 0.02, $p > 0.05$) however it showed significant increase in expression as compared to arsenic-treated group (0.24 ± 0.004, $p < 0.01$) (Figure 4.8 B).

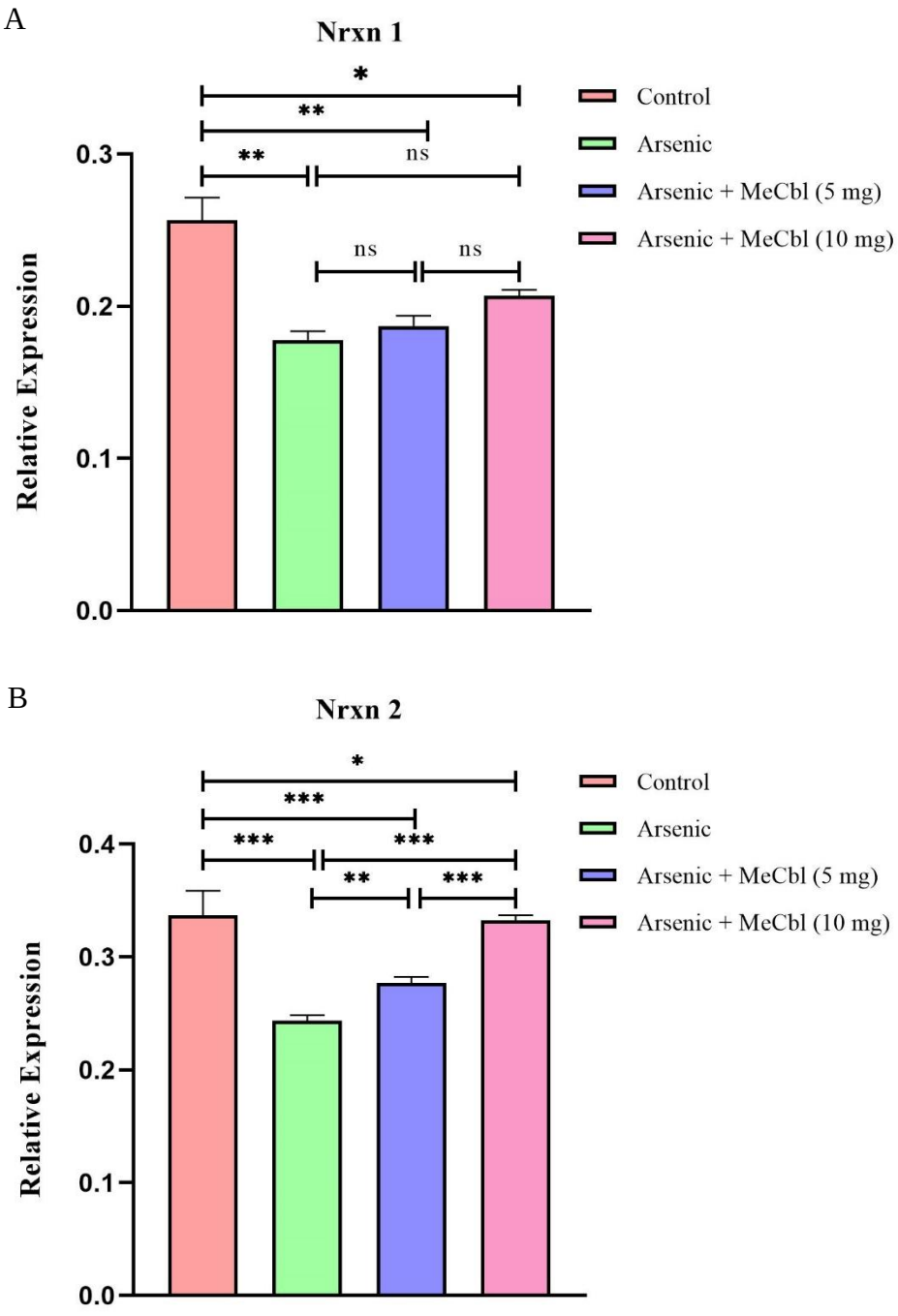


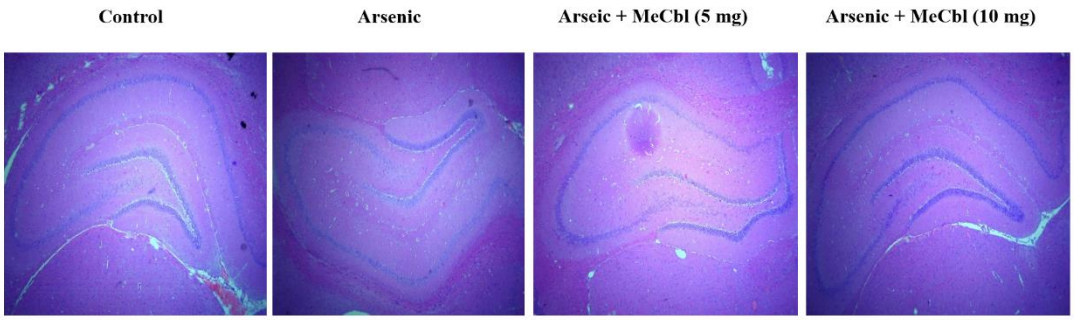
Figure 4.8: Transcriptional expression of hippocampal Nrxn 1 and Nrxn 2. Histogram representing (A) Nrxn 1 and (B) Nrxn 2 level for control group, arsenic, Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups (n=3). Mean ± SEM represented error bars using Graph pad Prism (Version 8.0.1) for One-way ANOVA, followed by Bonferroni's multiple comparison test. n.s= $p > 0.05$, *=$p < 0.05$, **= $p < 0.01$ and ***=$p < 0.001$. For consistency, duplicates of each sample were run.

4.3. Histological assessment of neuronal apoptosis post exposure to arsenic and treatment with MeCbl

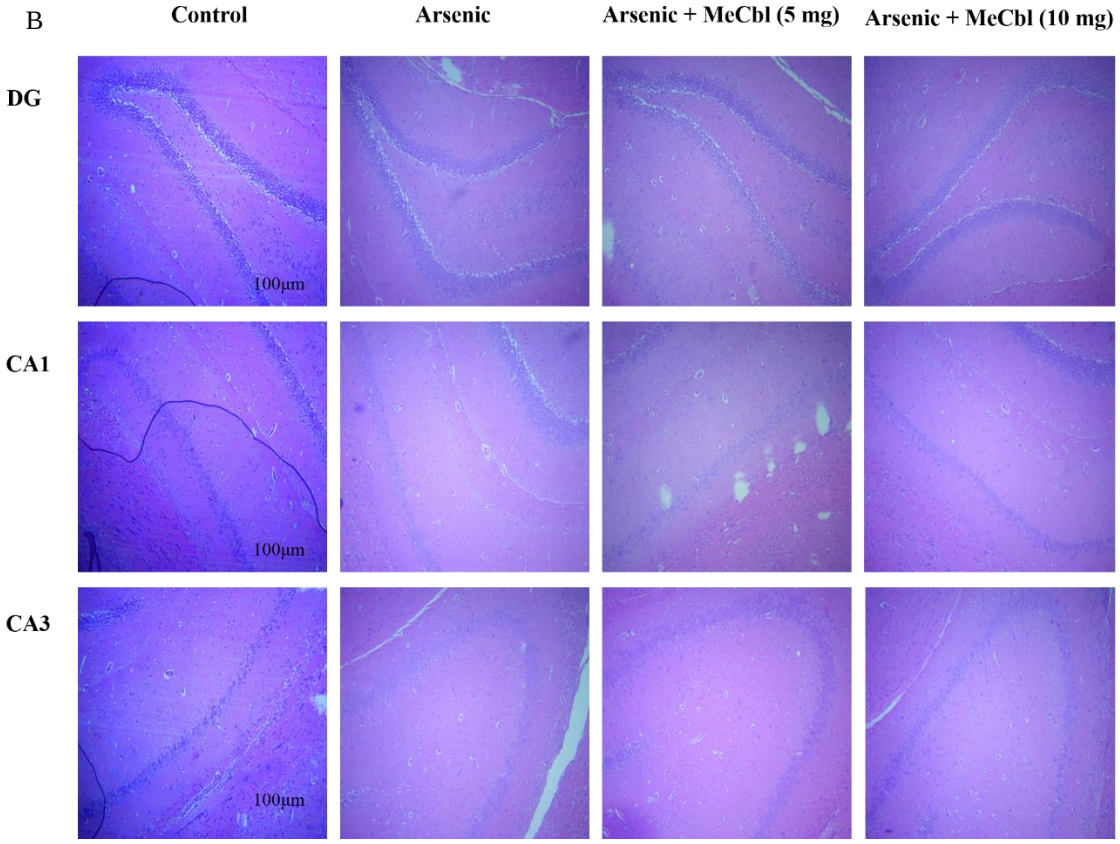
Histopathological assessment was performed in three areas of hippocampus (CA1, CA3 and DG) on transverse sections using H and E staining. Neuropathological alterations were observed in arseni**c**-treated group indicated by elongated irregular nucleus and shrunken cytoplasm. Control group exhibited normal morphology while Arsenic + MeCbl-treated group (10 mg) showed slight recovery in neuronal apoptosis. There was a substantial neuronal loss observed in arsenic-treated group (Figure 4.9 A, B, C).

H & E staining showed that both arsenic-treated and Arsenic + MeCbl-treated (5 mg) groups have significantly reduced number of cells in DG (26.5 ± 0.5, 28.15 ± 0.15), CA1 (10.4 ± 0.8, 11.7 ± 0.3) and CA3 (12.9 ± 0.1,13.9 ± 0.3) regions relative to cell number in DG (43.5 ± 1.5, $p < 0.001$ and $p < 0.01$), CA1 (17.5 ± 0.5, $p < 0.01$) and CA3 (20.8 ± 0.8, $p < 0.01$) regions of control hippocampus. Arsenic-treated and Arsenic + MeCbl-treated (5 mg) groups showed similar cell count in DG (26.5 ± 0.5, 28.15 ± 0.15), CA1 (10.4 ± 0.8, 11.7 ± 0.3) and CA3 (12.9 ± 0.1, 13.9 ± 0.3) regions of hippocampus. There was also comparable cell count in CA1 region of Arsenic + MeCbl-treated group rats (10 mg) (14.3 ± 0.3) relative to CA1 (17.5 ± 0.5, $p > 0.05$) region of control group hippocampus (Figure 4.10 A, B and C).

A


Control
Arsenic
Arseic + MeCbl (5 mg)
Arsenic + MeCbl (10 mg)

B


Control
Arsenic
Arsenic + MeCbl (5 mg)
Arsenic + MeCbl (10 mg)
DG
CA1
CA3
100µm
100µm
100µm

C

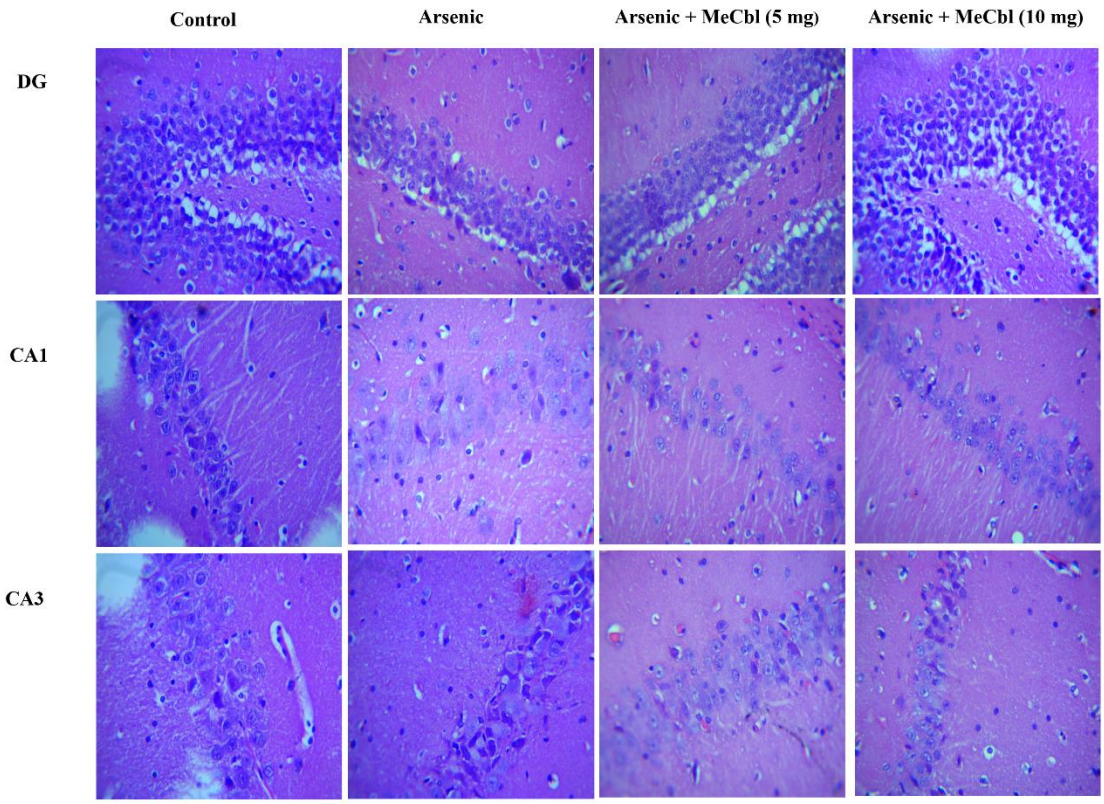


Figure 4.9: H&E staining at 4x (A) 10× (B) and (C) 40× magnification. Histopathological changes in three regions of hippocampus (CA1, CA3 and DG) are shown for control group, arsenic, Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups. Intact and circular shaped neurons with prominent nuclei in control group and lightly stained neurons of arsenic-treated group are shown in all studied regions of hippocampus. Neurons architecture were slightly recovered in Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups.

Arsenic + MeCbl-treated group (10 mg) showed slight reduction in number of cells in DG (37.5 ± 0.5) and CA3 (17.1 ± 0.1) regions in contrast to DG (43.5 ± 1.5, $p<0.05$) and CA3 (20.8 ± 0.8, $p<0.05$) regions of control group hippocampus but comparatively less reduction in neuronal cells and significant increase in number of cells in DG (37.5 ± 0.5) when compared to number of cells in DG (26.5 ± 0.5, 28.15 ± 0.15, $p < 0.01$) region of arsenic-treated and Arsenic + MeCbl-treated (5 mg) groups was observed. Arsenic +

MeCbl-treated group (10 mg) showed significant recovered cells in CA1 (14.3 ± 0.3) and CA3 (17.1 ± 0.1) regions in relation to CA1 (10.4 ± 0.8, $p < 0.05$) and CA3 (12.9 ± 0.1, $p < 0.05$) regions of arsenic-treated group hippocampus. Arsenic + MeCbl-treated group (5 mg) showed slight reduction in number of cells in CA1 (11.7 ± 0.3) region and no significant difference in CA3 (13.9 ± 0.3) regions as compared to number of cells in CA1 (14.3 ± 0.3) and CA3 (17.1 ± 0.1) regions of Arsenic + MeCbl-treated group (10 mg) hippocampus (Figure 4.10 A, B and C).

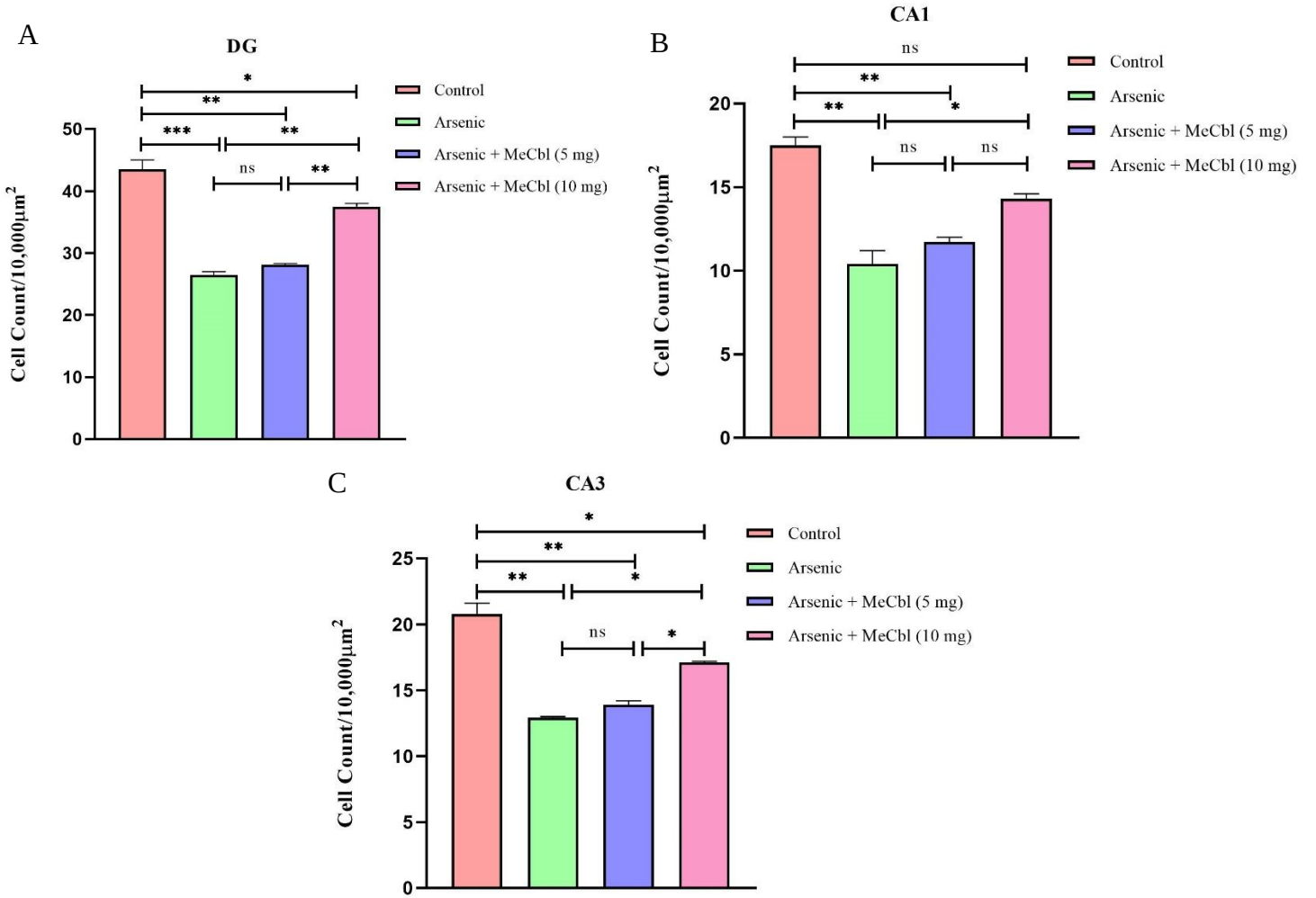


Figure 4.10: Histogram representing cell count/10,000 µm2 of the (A) DG, (B) CA1 and (C) CA3 for control group, arsenic, Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups. n.s = p > 0.05, *=p < 0.05, **= p < 0.01 and ***=p <

4.4. Impaired levels of antioxidants post exposure to arsenic and effect of MeCbl treatment

Brain oxidative stress status following arsenic and MeCbl treatment was determined by assessing hippocampal antioxidant enzyme activities (SOD, CAT, and GPx).

There was substantial decrease in the activity of SOD in arsenic-treated (2.49 ± 0.05) and Arsenic + MeCbl-treated (5 mg) (3.38 ± 0.02) groups when compared to control group (4.2 ± 0.10, $p < 0.001$) but activity of SOD was notably greater in Arsenic + MeCbl-treated group (5 mg) (3.38 ± 0.02) relative to arsenic-treated group (2.49 ± 0.05, $p < 0.001$). Arsenic + MeCbl-treated group (10 mg) (3.71 ± 0.02) showed significant decrease in the activity of SOD relative to control group (4.2 ± 0.10, $p < 0.01$)) but it showed considerably greater activity of SOD in relation to arsenic-treated group (2.49 ± 0.05, $p < 0.001$). There was significantly lower activity of SOD in Arsenic + MeCbl-treated group (5 mg) (3.38 ± 0.02) as compared to Arsenic + MeCbl-treated group (10 mg) (3.71 ± 0.02, $p < 0.05$) (Figure 4.11 A).

A significant reduction in activity of CAT was observed in both arsenic-treated (6.56 ± 0.17) and Arsenic + MeCbl-treated (5 mg) (6.31 ± 0.19) groups in contrast to control group (10.20 ± 0.26, $p < 0.001$) but similar activity of CAT in both treated groups ($p > 0.05$). Arsenic + MeCbl-treated group (10 mg) (9.07 ± 0.05) showed substantial decrease in the activity of SOD in contrast to control group (10.20 ± 0.26, $p < 0.05$) but it showed remarkably greater ($p < 0.001$) activity of SOD as compared to arsenic-treated (6.56 ± 0.17) and Arsenic + MeCbl-treated (5 mg) (6.31 ± 0.19) groups (Figure 4.11 B).

There was notable decrease in the activity of GPx in all treated groups relative to control group (9.76 ± 0.1, $p < 0.001$). Arsenic + MeCbl (5 mg) (8.77 ± 0.05) and Arsenic + MeCbl (10 mg) (9.1 ± 0.02) treated groups showed marked increase ($p < 0.001$) in activity of GPx when compared to arsenic-treated group (7.96 ± 0.07). The activity of GPx was also considerably greater ($p < 0.05$) in Arsenic + MeCbl-treated group (10 mg) (9.1 ± 0.02) relative to Arsenic + MeCbl-treated group (5 mg) (8.77 ± 0.05) (Figure 4.11 C).

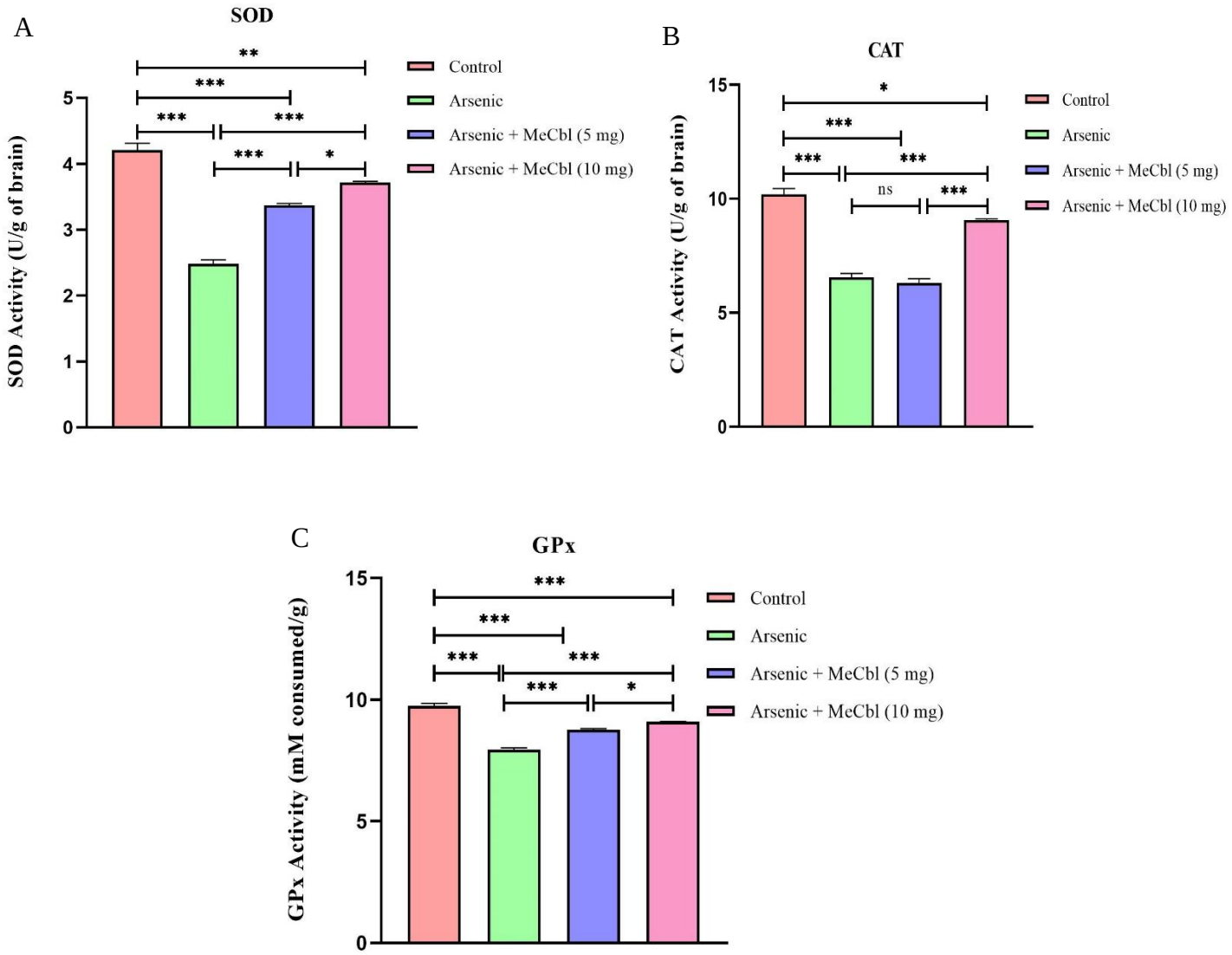


Figure 4.11: Histogram representing % activity of SOD, CAT and mM consumed/g of GPx for control group, arsenic, Arsenic + MeCbl (5 mg) and Arsenic + MeCbl (10 mg) treated groups. n.s = $p > 0.05$, *=$p < 0.05$, **= $p < 0.01$ and ***=$p < 0.001$ are the significance

CHAPTER 5

DISCUSSION

The current study was conducted to assess the therapeutic potential of two different doses of MeCbl on arsenic-induced changes in oxidative stress, synaptic plasticity and apoptosis in hippocampus of adult male wistar rats. Sodium arsenate (80 mg/kg) given for the period of two months, induced significant changes in the synaptic plasticity gene expression, antioxidant status and neurons survival while co-administration of MeCbl (5 mg) and MeCbl (10 mg) for similar period showed reversal of these changes.

Although, there is continuous production of ROS in the cells but it is depleted due to functioning of antioxidant system. ROS induced damage in the cells can be repaired by variety of antioxidants which control redox-sensitive signaling pathways. All mammals contain three primary antioxidant enzymes including CAT, substrate specific (GPx) and (SOD) in their oxygen metabolizing cells. The SODs provide molecular oxygen (O2) and hydrogen peroxide (by superoxide conversion) for CAT and GPx which transform these harmful species into oxygen and water. The net result is that two potentially harmful species, superoxide and hydrogen peroxide, are changed to water (Sharma et al., 2014).

Arsenic significantly brought down the CAT, SOD and GPx activities relative to the control and MeCbl at dose 10 mg/kg recovered the diminished level of antioxidant enzymes as compared to arsenic-treated rats (Figure 4.12). The finding is consistent with a study conducted by Peruru and Dodoala, (2021) in which arsenic treatment had considerably decrease the activity levels (GPx, CAT, and SOD) in relation to the normal control while treatment with diosmin at doses (50 and 100) mg/kg recovered the reduced antioxidant enzymes activities to normal. Similarly, another study showed that arsenic

exposure for 8 weeks caused a substantial decrease in SOD activity by 43%. Whereas administration of hydroxytyrosol alone did not stimulate SOD levels significantly however its simultaneous treatment with arsenic caused substantial increase in SOD activity equal to control (Soni et al., 2018). The modern approach for the treatment of arsenicism is the application of antioxidant to fight tissue damage due to arsenic persuaded OS (Baltaci et al., 2016; Sharma et al., 2018). Curcumin, catechin and quercetin are natural antioxidants having shielding effects against arsenic-induced neurotoxicity (Bhattacharya and Haldar, 2012). Despite the particular mechanism and pathology of neurotoxicity mediated by this metal is not well established however studies have reported that curcumin considerably reduces cholinergic dysfunction and exhibit neuroprotective efficiency against arsenic neurotoxicity (Jahanbazi Jahan-Abad et al., 2017). Protocatechuic acid has been reported to have neuroprotective effects on sodium arsenate induced toxicity in mice (Li et al., 2021). Melatonin has been approved effective against oxidative stress due to arsenic and inhibited apoptosis, inflammatory chemicals and DNA damage and it is proposed that melatonin could be a favorable and important therapy for the effective management of arsenicism in medical circumstances (Abdollahzade and Majidinia, 2021). As MeCbl has anticancer, antioxidant, inflammation inhibiting and anti-microbial activities. Thus, the present study explored the potential of MeCbl for neuroprotection against arsenic-induced neurotoxicity.

From the histological analysis, it was demonstrated that the cell numbers in subfields of hippocampus of rats exposed to arsenic were reduced relative to normal rats (Figure 4.10) and these results are in accordance with a study which showed that sodium arsenate modified architecture of nerve cell and their distribution in arsenic exposed brain

tissue sections (Li et al., 2021). Another study demonstrated that exposure of arsenic leads to increased markers of inflammation (pro-inflammatory) and oxidation (oxidative factors) along with damage to DNA and cell death in the nervous tissue of young rats in relation to controls ($p < 0.05$) (Abdollahzade and Majidinia, 2021). Simultaneous treatment with MeCbl (10 mg) improved the cell number in the hippocampus CA1 region which was comparable to control group (Figure 4.11 B) but no major difference was observed in other two regions of hippocampus (CA3 and DG) in all other groups although Arsenic + MeCbl (10 mg) treated group reversed the effects of arsenic-mediated neuronal loss and showed increase number of cells as compared to arsenic and Arsenic + MeCbl (5 mg) treated group (Figure 4.10). The neurodegeneration in arsenic group could be triggered by arsenic mediated oxidative stress and it was recovered by treatment with MeCbl (10 mg) and it showed the effectiveness of MeCbl (10 mg) as an anti-apoptotic agent (Figure 4.10).

Different mechanisms are involved for the accomplishment of antioxidant potential of MeCbl including uninterrupted foraging of ROS, principally mitochondrial and cytosol superoxide (Chan et al., 2018) and secondarily exciting ROS foraging by maintenance of glutathione, SOD and GPx (Lauer et al., 2022; Karamshetty et al., 2018). Ascorbic acid has already been described as an antioxidant against neurotoxicity induced by lead (Karamian et al., 2015). MeCbl can be effective to cure neurotoxicity induced by glutamate. MeCbl and estradiol benzoate have been reported to have role in the treatment of nervous disorders (memory problems and multiple sclerosis) through activation of the hippocampal proteins (Taherian et al., 2021). Hippocampus cells, accountable for memory and learning are upset in AD, causing the production of numerous inflammatory markers (COX–2, Nrxn 1, Nlgn, and caspase–3 proteins) used in measurement of AD development

and severity. MeCbl having role in brain functioning possess anti–inflammatory properties and neuron cell death in AD is associated with its impairment. MeCbl has been reported to significantly improve molecular equilibrium in scopolamine–induced AD in rats and restored the synaptic plasticity. PSD–95 and Nrxn 1 and Nlgn concentrations were considerably reduced, whereas activated caspase-3 and COX-2 was boosted in the hippocampus of scopolamine–inoculated rats. Cbl administration decreased these alterations and repressed hippocampal apoptosis and inflammation with the preservation of proteins (pre– and post–synaptic) and probably hippocampal synaptic integrity (Mehrdad et al., 2021). In addition, clinical studies consistently displayed that MeCbl alone or along with vitamin B family additional representatives have positive properties for brain atrophy, cognitive function and infection and in aged people without cognitive impairment or in slight cognitive loss patients. Patients having AD were found to have reduced plasma level of MeCbl in comparison of healthy control while B vitamins supplementation improved cognition in several clinical trials and in in many reported studies (Lauer et al., 2022). In the present work besides Nlgn 2 and Homer, all synaptic plasticity markers were downregulated due to arsenic exposure. The downregulation of synaptic plasticity genes was correlated with previous studies conducted showing the negative effects of heavy metals on synaptic plasticity. No study was reported before for therapeutic effects of MeCbl on pre- and post-synaptic markers alterations induced due to arsenic. Although arsenic model for synaptic plasticity genetic expression changes has not been developed in previous research but exposure to other neurotoxicants having decreased expression of PSD-95 has already been evaluated. Prenatal bisphenol exposure in mice reduced expression of PSD-95, spatial and aversive memory impairment, anxiety-related behavior

(Wang et al., 2021) and mice exposure to methylmercury is associated with declining expression of syntaxin and PSD -95 with reduction in neurons branching and dendritic length (Wang et al., 2022). Sevoflourane exposure in neonatal rats also reduced hippocampal synapse density, fear memory, radically decreased PSD-95 and kalirin-7 expression in the hippocampus during developmental period (Li et al., 2022) and it is consistant with the decreasing expression induced by arsenic and significantly increased expression with MeCbl (10 mg) and MeCbl (5 mg) treatment simultaneously with arsenic although not comparable to control (Figure 4.2). Nitrite exposure in mice decreased cortical SYN positive buttons (Chen et al., 2015) while trimethyltin exposure reduces the level of hippocampal SYN protein (Zhang et al., 2014) and in current study, arsenic reduced the SYN genetic expression. It is observed in the current study that MeCbl (10 mg) treatment was not sufficient to revive it completely. Sevoflourane exposure in neonatal rats reduced hippocampal synapse density, fear memory, and radically decreased the gene expression of kalirin-7 in the developing brain hippocampus (Li et al., 2022) and 80 mg/kg of sodium arsenate was sufficient to decrease its expression without any revival with both doses of MeCbl (Figure 4.3 B). Sevoflurane (3%) exposure in newborn rats for 6 h in rats elevated expression of hippocampal SNAP-25 (Xiao et al., 2016) and acrylamide exposure in rats caused downregulation of SNAP-25 in cortex and hippocampus along with neuronal necrosis (Fang et al., 2022). Arsenic exposure in balb/c mice has role in SNAP-25 and PSD-95 expression reduction leading to impaired memory and learning (Ansari et al., 2022) as described in our study although reduction in expression was more significant in Arsenic + MeCbl (10 mg) treated group in comparison to arsenic treated group (Figure 4.4 A). Disrupted synaptic plasticity along with impairment of memory for long period are the

result of CAMK-IV knockout in mice (Song et al., 2015; Wang et al., 2021). Arsenic effect on repressing cerebellar long term depression formation, along with worse task performance for spatial memory by decreasing the expression of CAMK-IV has been worked (Ghosh and Giese, 2015; Guan et al., 2016) and this is consistent with the result of our study in which sodium arsenate significantly decreased the expression of CAMK-IV to the level which was not able to be recovered with MeCbl (5 mg) and showed a little increase in the expression when treated with MeCbl (10 mg) showing that 10 mg of MeCbl could be able for reversal of changes in expression (Figure 4.4 B). Lead exposure in offspring rats reduced Homer1 expression leading to learning and memory impairment due to abnormal spine density (Pang et al., 2019). In homer1 knockout mice, hippocampal Homer1c upregulation has been able to save memory and learning ability (Gimse et al., 2014). This is opposite to the present study in which expression was unaffected among all groups showing that arsenic did not affect the homer gene expression (Figure 4.5). Ex-vivo exposure to a bio metal profile for autism spectrum disorder reduced shank 1 and shank 2 expression along with a abnormal density of synapsis (Hagmeyer et al., 2015), whereas lanthanum exposure in offspring rats has been reported to cause significant reduction in expressions of both mRNA and proteins including SYN, PSD-95, Shank 3, synapsin and Shank 1 in the hippocampus with reduction in memory and learning ability (Ding et al., 2021). The present study observed that both shank 1 and shank 2 showed decreased expression due to sodium arsenate with reversal of shank 2 expression with MeCbl (10 mg) treatment (Figure 4.6). It is evident that lead exposure down-regulates the hippocampal Nlgn 1 protein levels with altered spine plasticity during development (Zhao et al., 2018). Another intersting study, reported the methamphetamine exposure with increased PSD-95,

Nlgn 1 and synapsin 1 expression in rats (Cao et al., 2022). Similarly, the 6-hydroxydopamine leisons reduced hippocamapal PSD-95, Nlgn 1 and Nrxn1 expression level in rats (Sheibani et al., 2022). Bisphenol-A exposure in rodents impaired spatial memory by upregulated expression of Nrxn1 with increased hippocampal and cortical dendritic spine density (Kumar & Thakur., 2014). The present study also observed that arsenic decreases the expression of Nlgn 2 as compared to control but less reduction in expression as compared to both Arsenic + MeCbl-treated groups (Figure 4.7 B). The Nlgn 1 expression was more reduced in Arsenic + MeCbl (5 mg) treated group relative to other treated rats indicating the nagative effects of this dose of MeCbl (Figure 4.7 A).

Overall current study highlighted that arsenic treatment induced more significant downregulation of SYN, PSD-95, Shank2, Nrxn 2, Nlgn 1, Kalirin and CAMK-IV expression and less significant downregulation in the expression of Nrxn 1 and Shank 1. The expression of Nlgn 2 and SNAP-25 was only slightly reduced and expression of homer was alike among all study groups. Arsenic treatment induced oxidative stress leading to substantial decrease in antioxidant activity (SOD, CAT and GPx). Histopathological examination revealed a remarkably lower cell count in hippocampus (DG, CA1 and CA3) after arsenic treatment. Simultaneous treatment with MeCbl (10 mg) resulted in positive shift in the expression of Syn, Shank 1, Shank 2, Nrxn 2, PSD-95, kalirin and CAMK-IV while the expression of Nlgn 2, SNAP-25 and Nlgn 2 was lower than arsenic treated group. There was increased antioxidant enzymes activity (SOD, CAT and GPx) in rats treated with Arsenic + MeCbl relative to arsenic treated rats and CAT activity was less in Arsenic + MeCbl (5 mg) treated rats. Cell count in hippocampus (DG and CA3 regions) was also increased in both Arsenic + MeCbl treated group rats but only Arsenic + MeCbl (10 mg)

treated rats were able to recover the cell density in CA1 region of hippocampus. The findings of this study suggest that MeCbl (10 mg) could be effective option to reverse the changes in synaptic plasticity, histopathology and antioxidant status induced by arsenic and to recover downregulation in most of the synaptic gene expression to some extent as compared to MeCbl (5 mg).

CONCLUSIONS

Sodium arsenate exposure significantly affects the activity of antioxidant enzymes i.e., CAT, SOD and GPx as compared to the control group while MeCbl at dose 10 mg reinstituted the diminished level of antioxidant enzymes as well as changes in histopathology as compared to sodium arsenate treated rats. However, the synaptic gene expression with simultaneous treatment with MeCbl do not completely reverse the effects. It is concluded that MeCbl (10 mg/kg) could be an effective option to reverse the arsenic-induced alterations in synaptic plasticity, neuronal density, antioxidant levels and gene expression.

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