AMELIORATIVE EFFECTS OF ASCORBIC ACID ON NEUROBEHAVIOURAL, HAEMATOLOGICAL AND BIOCHEMICAL CHANGES INDUCED BY SUB-CHRONIC LEAD EXPOSURE IN WISTAR RATS

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DECLARATION

I declare that the work in the thesis, entitled "Ameliorative Effects of Ascorbic Acid on Neurobehavioural, Haematological and Biochemical Changes Induced by Sub-chronic Lead Exposure in Wistar Rats" has been performed by me in the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria under the supervision of Professors J. O. Ayo, M. Mamman and S. A. Ojo. The information derived from the literature has been duly acknowledged in the text and in the list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

_____ _____

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CERTIFICATION

This thesis, entitled "Ameliorative Effects of Ascorbic Acid on Neurobehavioural, Haematological and Biochemical Changes Induced by Sub-Chronic Lead Exposure in Wistar Rats" by Oladipo, Olusola Olalekan meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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This project is dedicated to God Almighty, my help in ages past.

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ABSTRACT

Experiments were performed with the aim of evaluating the effects of sub-chronic exposure to lead and the protective role of ascorbic acid on toxic changes in Wistar rats. Forty adult Wistar rats (20 males and 20 females) divided into 4 groups of 10 animals each (5 males and 5 females in each group) served as subjects for this study. Rats in group I were given distilled water only. Rats in group II were exposed to lead acetate only at 200 mg/kg, while those in group III were pre-treated with ascorbic acid only at a dose of 100 mg/kg. Rats in group IV were pre-treated with ascorbic acid at a dose of 100 mg/kg before being administered with lead acetate 30 minutes later. The regimens were administered orally once daily for a period of six weeks. The effects of the different treatments on body weight change, open-field activities, sensorimotor reflexes and cognition were evaluated in the rats. Packed cell volume (PCV), haemoglobin concentration (Hb), total erythrocyte count (RBC), total leucocyte count, differential leucocyte count and erythrocyte osmotic fragility (EOF) test were determined using standard methods. Total protein, albumin and globulin concentrations, and activities of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and creatine kinase (CK) were measured using standard methods. Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substance; malondialdehyde (MDA) in the brain of the rats. Serum thyroid hormone levels were also determined using radioimmunoassay. The results demonstrated that sub-chronic exposure to lead in rats had no effect on body weight change. There was no significant ($P > 0.05$) change in sensorimotor reflexes of the rats. Rats in the lead-treated group showed an increased anxiety compared to the control and those pre-treated with ascorbic acid. Rats in the lead group also showed a significant ($P < 0.05$) decline in cognitive function compared to those in the control and ascorbic acid-pretreated groups. The haematology showed significant ($P < 0.05$) reductions in PCV, Hb and RBC in the lead-

treated group compared to the control rats. Lead also caused leucocytosis due to a significantly ($P < 0.05$) higher neutrophil/lymphocyte ratio. There was slight but insignificant increase in EOF in rats exposed to lead, compared with the control groups. A significant ($P < 0.05$) decrease in AST, LDH, CK and a slight increase in MDA was observed in the lead treated rats compared to the control groups. There were tissue changes in the lead-treated group compared with the control and the ascorbic acid pre-treated groups. The study demonstrated that increased lipid peroxidation, low thyroid indices and decreased CK activity may be involved in the mechanism of neurobehavioural and cognitive impairments by lead. In conclusion, the study showed that sub-chronic exposure to lead induced anxiogenic effects, cognitive, haematological, biochemical and pathological changes in rats, ameliorated by pretreatment with ascorbic acid. It is, therefore, recommended that individuals, who are constantly exposed to lead, should be pretreated with vitamin C.

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LIST OF ABBREVIATIONS

8-OHdG	8-hydrodeoxyguanosine
AA	Ascorbic acid
ALA	Aminolevulinic acid
ALAD	Aminolevulinic acid dehydratase
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid
ANOVA	Analysis of variance
AP-1	Apoptotic factor 1
$AscH^-$	Ascorbate ion
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
ATSDR	Agency for toxic substances and disease registry
BAL	British antilewisite
Ca^{2+}	Calcium ion
C-H	Carbon-hydrogen bonds
CK	Creatine kinase
CLS	Chemiluminiscent spectroscopy
CoA	Co-factor A
DHA	Dehydroascorbic acid
DMSA	Meso- 2, 3 -dimercaptosuccinic acid
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diamine tetraacetic acid
EDTA	Ethylene diaminetetraacetic acid

ERS	Electromagnetic resonance spectroscopy
Fe^{2+}	Oxidized iron
Fe^{3+}	Reduced iron
GMT	Greemwich mean time
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H & E	Haematoxylin and eosin
Hb	Haemoglobin
HepG2	Human carcinoma cells
IQ	Intelligence quotient
LD_{50}	Mean lethal dose 50
LDH	Lactate dehydrogenase
MDA	Malondialdehyde
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NFkB	Necrotic factor kB
NMDA	N-methyl-D-aspartate
OD	Optical density
Pb^{2+}	Lead ion
PCV	Packed cell volume
PKC	Protein kinase C
PUFA	Polyunsaturated fatty acid
RBC	Total erythrocyte counts
RNA	Ribose nucleic acid
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
Se	Selenium
SEM	Standard error of the mean
SH	Sulfhydryl group
T_3	Triiodothyronine
T_4	Thyroxine
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TNF-α	Tissue necrotic factor
TSH	Thyroid stimulating hormone
USEPA	United states environmental protection agency
Zn^{2+}	Zinc ion

CHAPTER ONE

1.0 INTRODUCTION

Lead is a well-known environmental toxicant that has been used since ancient times as a component of domestic, agricultural and industrial products. Today, this heavy metal is one of the ubiquitous environmental pollutants, particularly widespread in industrial areas. This metal is primarily found in leaded gasoline, paints, house dusts, pesticides, and some industrial products, such as glazes, colouring materials for ceramics, hose and pipes (Srianujata, 1997; Tong *et al.*, 2000). In many developed countries, lead has been eliminated from paint and gasoline, but some developing countries like Nigeria, still make use of leaded fuel. In addition, citizens of big cities have higher risk of environmental exposure to lead than do inhabitants of rural areas (Srianujata, 1997).

The use of lead in developing countries such as Nigeria is still a major problem (Igwilo *et al.*, 2006; Nduka and Orisakwe, 2007). Man and animals are exposed to lead through feed from numerous sources, as well as from the general environment. Lead is still being used in our environment as components of lead fuel, in paints, cosmetics, agricultural pesticides; and even leaches from battery industries and mechanic workshops find their way into the water beds, thereby contaminating the water bodies. These leaches enter into the food chain; accumulate in body tissues of animals and humans, resulting in toxicity. In a study conducted in the city of Jos, Nigeria, risk factors such as the use of lead-containing eye cosmetics, chipping paint in the home and proximity to battery smelters and gasoline sellers have been identified as the major sources of high blood lead levels amongst the inhabitants (Wright *et al.*, 2005). Lead poisoning is an insidious poisoning that causes the impairment of learning and memory (Aldridge *et al.*, 2005; Haider *et al.*, 2005). In fact, low intelligent

quotient amongst individuals has been associated with chronic lead exposure (Aykin-Burns *et al.,* 2003).

Ingested lead has resulted in poisoning, poor performance and death in animals and man (McDowell, 1992; Gurer and Ercal, 2000). Accumulated lead in tissues is toxic in most of its chemical forms, whether it is inhaled or ingested in water or feed. In fact, due to its slow rate of elimination, harmful levels of lead accumulate in tissues after prolonged exposure to low quantities (Demichele, 1984; Ercal *et al.,* 2001). Lead has also been shown to produce a wide range of acute and chronic effects and induces a wide range of physiological, biochemical and behavioural dysfunctions. It affects membranes and biochemical processes and has been shown to induce oxidative stress by acting as a catalyst in the oxidative reaction of biological molecules (Ercal *et al.,* 2001; Galaris and Evangelou, 2001; Gurer and Ercal, 2000; Aykin-Burns *et al.,* 2003).

The toxicities associated with lead have been attributed to oxidative tissue damage (Aykin-Burns *et al.,* 2003; Patrick, 2006). Under normal conditions, there are protective mechanisms *in vivo* to prevent peroxidative tissue damage. They include free-radical scavengers (antioxidants) and enzymatic defense systems (antioxidant enzymes). Ascorbic acid is a well-known non-enzymatic antioxidant involved in several biochemical processes in biological systems. This vitamin breaks the chain of lipid peroxidation in cell membranes and scavenges free-radicals such as superoxide ion, hydrogen peroxide, nitric oxide, hydroxyl, alkoxyl and alkyl-peroxyl, collectively called reactive oxygen species (ROS) (Carr and Frei, 1999; Kacuk *et al.,* 2003).

1.1 Statement of the Problem

There are few regulations (WHO, USEPA, etc) on lead; hence sources of lead exposure still abound without control. Also, lead is slowly eliminated from the body and bio-

accumulates in vital tissues of the body. In a multi-city study by Lanphaer *et al.* (2005), a biphasic intelligence quotient (IQ) dose-response curve showed steeper changes in IQ at very low blood lead level. This situation puts animals and man at risk of decreasing IQ, and they may need extra-assistance, especially animals whose cognitive function is being put to task. Also, most of the neuro-behavioural dysfunctions resulting from lead poisoning have been reported to be irreversible (Needleman, 1990; Fisher and Vessey, 1998). This may pose social and economic problems to the individuals and governments concerned. In addition, alternative biomarkers for lead neuro-toxicity/exposure, apart from delta-aminolevulinic acid (ALA) in serum/urine have not been fully elucidated.

1.2 Justification

Despite extensive research on heavy metals toxicity, relatively little attention has been paid to the modulating effects of vitamins and possible implications of nutritional status on heavy metals exposure in humans and animals. Apparently, the deleterious effects of lead on neurobehavioural, haematological and biochemical parameters in man and animals might be modified by ascorbic acid supplementation. The clinical and experimental data, in the literature, in support of this theory are still limited.

1.3 General Aims of the Study

The aim of this study was to investigate the changes occurring in rats exposed to low-level lead poisoning, and the protective effect of ascorbic acid on the poisoning.

1.4 Specific Objectives of the Study

1. To investigate the effect of repeated low-dose lead exposure on behavioural and cognitive changes in Wistar rats.

2. To investigate the effect of repeated low-dose exposure to lead on some haematological and biochemical parameters, and tissue pathological changes.
3. To determine if the behavioural, cognitive, haematological, serum biochemical and tissue pathological changes induced by low-dose administration of lead can be ameliorated by ascorbic acid.

1.5 Statement of Hypotheses

1. **Null hypothesis:** Ascorbic acid does not have any ameliorative effects on low-level lead poisoning.
2. **Alternate hypothesis:** Ascorbic acid has ameliorative effects on low-level lead poisoning.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Lead

2.1.1 Historical perspectives and use

Lead is a well-known metal from time immemorial. It has an atomic weight of 207.2, atomic number of 82, melting point of 327.502^0C, boiling point of 1740^0C, specific gravity of 11.5 and valence of +2 or +4 (Weast *et al*., 1986). The alchemists believed that lead is the oldest metal and associated it with the planet Saturn. Native lead occurs in nature, but it is rare. Lead is obtained chiefly from Galena (PbS) by a roasting process. Anglesite ($PbSO_4$), Cerussite ($PbCO_3$), and Minim (Pb_3O_4) are other common lead minerals (Weast *et al*., 1986).

Warnings of lead's poisonous properties extend as far back as the second century B.C., when Nikander, a Greek physician, described the colic and paralysis that followed lead ingestion. The early victims of lead toxicity were mainly lead workers and wine drinkers. The sweet flavour of lead made it to be of value in wine-making, to counteract the astringent flavour of tannic acid in grapes (Needleman, 2004). In fact, lead-sweetened wine contains as much as 20 mg of lead per litre. Wide-spread outbreaks of lead colic continued in Europe until as late as the sixteenth century, when the German physician, Eberhard Gockel, traced a colic epidemic to lead-adulterated wine. Duke Ludwig of Wurtemberg, upon learning of an epidemic of lead colic in his duchy, banned its use in wine-making and imposed death penalty for violators (Needleman, 2004).

Lead pipes bearing the insignia of Roman emperors used as drains from the baths are still being used today in old buildings. Lead is used in containers for corrosive liquids (such as in sulphuric acid chamber), and may be toughened by the addition of a small percentage of antimony of other metals. Natural lead is a mixture of three stable isotopes: Pb^{204} (1.48%),

Pb^{206} (23.6%) and Pb^{208} (52.3%) (Weast *et al*., 1986). Great quantities of lead, both as the metal and as the dioxide, are used in storage batteries. Much of the metal also goes into cable covering, plumbing, ammunition and in the manufacture of lead tetraethyl, used as an antiknock compound in gasoline. The metal is very effective as a sound absorber; therefore, it is used as a radiation shield around X-ray equipment and nuclear reactors to absorb vibration (Fischbein, 1992).

Lead has a number of uses, many of which are currently being phased out because of growing awareness of its toxicity and the damage that its uncontrolled dispersion in the environment has caused (ATSDR, 1993). White lead, the basic carbonate, sublimed white lead ($PbSO_4$), chrome yellow ($PbCrO_4$), and other lead compounds are used extensively in paints, although, in recent years, the use of lead in paints has been drastically curtailed to eliminate or reduce health hazards (Weast *et al*., 1986). Lead oxide is used in producing fine "crystal glass" and "flint glass" of a high index of refraction for achromatic lenses. It is also used in X-ray protective jackets. The nitrite and the acetate (Figure 1) are soluble salts. Lead salts such as lead arsenate have been used as insecticides, but their use in recent years has been practically eliminated in favour of less harmful organic compounds (ATSDR, 1993). Environmental concern with lead poisoning has led to the development of a national programme in the U.S.A. to reduce the concentration of lead in gasoline (USEPA, 1996). Such a programme is not being fully implemented in many developing countries, including Nigeria.

2.1.2 Histopathological changes of tissues following lead poisoning

Post-mortem changes characteristics of lead poisoning in a case of cattle exposed to ash residues contaminated with lead, revealed cerebro-cortical oedema, cortical neuronal necrosis and endothelial proliferation, especially at the tips of the cerebral gyri (Krametter-

Froetscher *et al*., 2007). Michaelson and Sauerhoff (1974) also found oedema and gross vascular changes in the cerebellum following lead exposure in neonatal rats. Demyelinated neuropathy has been reported in chronic lead poisoning (Gelman *et al.,* 1987). Intranuclear inclusions in epithelial cells lining the renal proximal tubules of rats have been detected following lead exposure (Murakami *et al.,* 1983). McMurry *et al.* (1995) demonstrated altered renal proximal tubular epithelium, renal intranuclear inclusions and decrease numbers of sperm and developing follicles in rats. These changes were reported to be more pronounced with increased lead concentration and duration of exposure. Alterations in the synovium and bone of individuals with lead shot wounds showed formation of nuclear lead inclusions, dilation and degranulation of the rough endoplasmic reticulum and deposition of crystalline precipitates in the matrix of the mitochondria in macrophages, osteoclasts and synoviocytes as well as the development of cytoplasmic lead inclusions in osteoclasts (Slavin *et al.,* 1988).

2.1.3 Haematological changes occurring following lead poisoning

Decrease in total erythrocyte counts following exposure to lead has been reported in fish (Shah, 2006; Ates *et al.,* 2008), mice (Iavicoli *et al.,* 2003) and rats (Ahamed *et al.,* 2007; Jadhav *et al.,* 2007). Other haematologic parameters such as haemoglobin concentration, packed cell volume and mean corpuscular haemoglobin concentration were shown to decrease in lead exposed individuals (Shaik and Jamil, 2009) and animal models (Shah, 2006; Ahamed *et al.,* 2007; Jadhav *et al.,* 2007; Ates *et al.,* 2008). The immune function has also been demonstrated to be sensitive to lead exposure (Basaran and Undeger, 2000; Mishra *et al.,* 2003). McMurry *et al.* (1995) reported alterations in total leucocyte counts, lymphocytes, neutrophils, eosinophils and total splenocyte yield after subchronic exposure to lead in cotton rats (*Sigmodon hipidus*).

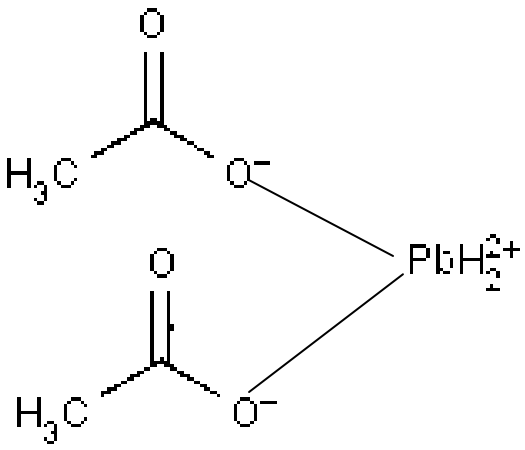


Figure 2.1. Chemical structure of lead acetate.

2.1.4 Mechanisms of toxicity

Lead (Pb^{2+}), a heavy metal, has been used by humans for many technological purposes, which is the main reason for its present widespread distribution. Although various actions have been taken to decrease the use, distribution and pollution of lead in the environment, its environmental pollution remains a significant health hazard throughout the world. Several mechanisms of lead poisoning have been identified. The most common are pica, industrial exposure, drinking moonshine liquor, inhalation, gun-shot wounds, retained lead pellets or particles, and a variety of folk remedies and cosmetics (Pfitzner *et al*., 2000).

The toxic mechanism of lead is caused by its ability to substitute for other polyvalent cations (particularly divalent cations, such as calcium [Ca^{2+}] and zinc [Zn^{2+}] in the molecular machinery of living organisms. These interactions allow lead to affect different biologically significant processes, including metal transport, energy metabolism, apoptosis, ionic conduction, cell adhesion, inter- and intracellular signalling, diverse enzymatic processes,

protein maturation, and genetic regulation. Membrane ionic channels and signalling molecules seem to be one of the most relevant molecular targets contributing to neurotoxicity induced by lead (Garza *et al*., 2006). Once it has penetrated the cytoplasm, lead continues its destructive mimicking action by occupying the calcium-dependent proteins. It binds to calmodulin, a protein which in the synaptic terminal acts as a sensor of free calcium and as a mediator of neurotransmitter release. Furthermore, it alters the functioning of the enzyme protein kinase C, a virtually ubiquitous protein which is of crucial importance in numerous physiological functions. Protein kinase C is normally activated by modulators outside the cell (hormones, neurotransmitters) through an enzyme chain and in a calcium-dependent manner. Besides many other functions, the activated kinase directly affects the expression of the immediate early response genes (Bressler *et al*., 1999).

Lead has high affinity for the sites which are typical calcium-binding sites in this protein; picomolar doses can take the place of micromolar calcium doses. In model cell systems, it has been demonstrated that lead can stimulate gene expression through a mechanism mediated by protein kinase C, and it is postulated that this effect may be correlated with alterations in synaptic functioning (Bressler *et al*., 1999). The mechanism involved in rat brain neurotoxicity by lead has been demonstrated to be via the induction of oxidative stress as a result of the accumulation of delta-aminoalevulinic acid (ALA) during lead exposure (Wang *et al*. 2006). ALA selectively inhibits gamma-aminobutyric acid (GABA) release from synaptosomes and blocks GABA receptors (Wang *et al*., 2006). Mateo *et al*. (2003) also reported an increase in acetylcholinesterase activities following lead exposure. Lead also causes prolong rise time of the trans-membrane potential at nodes of Ranvier, thereby altering nerve conduction time (Krieg *et al.*, 2008). Furthermore, the disturbances caused by lead can be attributed to increase in reactive oxygen species,

especially in the brain. The brain has a high rate of oxidative metabolism and high content of unsaturated lipids (Bennet *et al.*, 2007)

2.1.5 Pathophysiology of lead poisoning

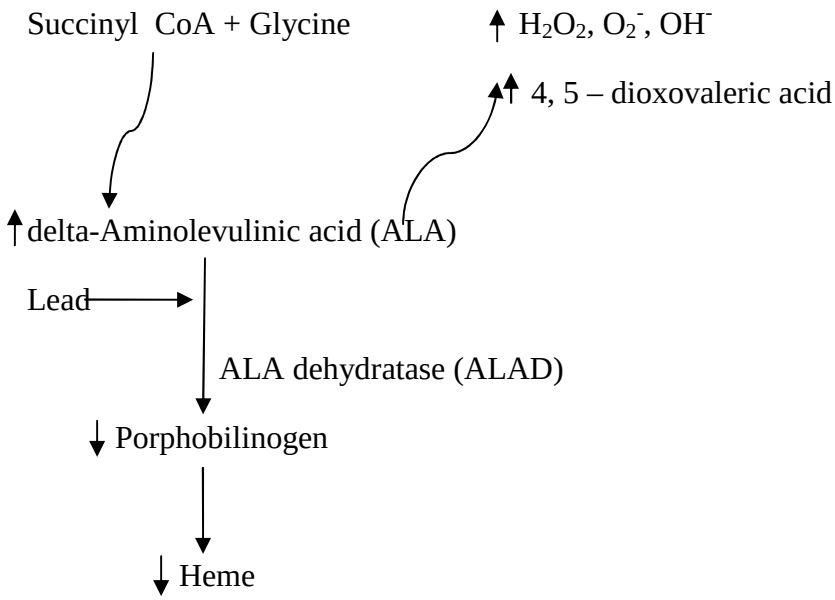
Lead ranks second in the list of prioritised hazardous substances issued by the U.S. agency for toxic substances and disease registry (ATSDR, 2005). The noxious effects of this metal have long been well-known, especially as regards acute forms of poisoning. Chronic lead poisoning was initially defined by blood lead levels above 80 µg/dl, but today a lead level of 30 µg/dl in blood is considered excessive and levels at or above 10µg/dl (0.1ppm) are considered potentially harmful, particularly to juveniles (Lidsky and Schneider, 2003).

Once absorbed by the body, mainly through breathing and feeding, lead is not metabolised, but mostly expelled. The remaining portion (about 20%) settles into the tissues and notably:

1. In the blood, where it is carried almost exclusively by the erythrocytes;
2. In mineral tissues (bones and teeth), where it deposits; and
3. In soft tissues (kidneys, bone marrow, liver and brain) (Leggett, 1993).

Animal studies have shown that certain substances bind lead and increase its solubility, thereby enhancing its absorption. Sodium citrate, amino acids, vitamin D, protein and fat, and lactose increase lead solubility and, hence, its absorption. Bile is an important route of excretion in the gut. The primary site of lead absorption is the duodenum, where it enters the epithelial mucosal cells (Merrill *et al.*, 2008). The total amount of lead in the body does not affect absorption or have a negative feed-back mechanism that limits absorption of lead.

The presence of lead in the blood stream (inside the red blood cells and mostly linked to haemoglobin) provokes anaemia (Bhambhani and Aronow, 1990; Muwakkit *et al.,* 2008). This cannot be considered a symptom, but rather a delayed sign of lead poisoning (Figure 2.2). Through the blood, lead reaches all other tissues. In view of its capacity to "mimic" calcium, lead is stored in the bones and becomes a stable bone component, particularly in the case of insufficient calcium intake (Hipkins *et al.,* 1998). This lead deposit may be mobilised and returned into the blood stream under particular states of physiological (pregnancy, breast-feeding) and pathological stresses (diseases), but also as a consequence of greater calcium intake in the diet (Hu *et al.*, 1990). This stable presence of lead in bones makes recovery from lead poisoning extremely slow, even when the toxic agent has been completely eliminated (Gruber *et al.,* 1999; Gu *et al*., 2005).



Key: ↑ or ↓ indicate changes in enzymes or substrates as a result of lead exposure.

Figure 2.2: Inhibition of delta-Aminolevulinic acid dehydratase results in elevated delta-aminolevulinic acid. (Patrick, 2006).

Chronic lead nephropathy or chronic tubule-interstitial nephritis occurs in long-term lead exposure, and is often associated with hypertension and gout (Hipkins *et al.*, 1998). Lead nephropathy is recognized in three forms. First is acute lead poisoning due to an acute, massive exposure to lead. This causes the classic symptoms of colic, encephalopathy, anaemia and neuropathy (Hertz-Picciotto and Croft, 1993). Second is chronic lead nephropathy, a slowly progressive interstitial nephritis due to an excessive cumulative, exposure to lead. This is often associated with hypertension and gout and as at today more difficult to diagnose because there are no detectable laboratory changes of acute lead intoxication. The typical clinical picture and the exclusion of other causes of renal disease aid in its diagnosis. The third form is lead hypertension, which results from hepatic and kidney damage; thus causing and imbalance in ion homeostasis (Harlan, 1988).

2.1.6 Treatment for lead poisoning

Treatment for lead poisoning relies on drugs that have a chelating effect on the metal; these are molecules that can bind to lead and form a stable complex, which is then expelled. The most common drugs used are calcium-disodium ethylene diamine tetraacetic acid ($CaNa_2EDTA$), Meso-2, 3-dimercaptosuccinic acid (DMSA or British antilewisite [BAL]) and penicillamine, a penicillin derivative which has a chelating, rather than anti-bacterial effect (Fisher and Vessey, 1998; Torres-Alanis *et al.*, 2002). $CaNa_2EDTA$ is a water-soluble chelator, and is readily excreted, and administered parenterally for 3-5 days in children exposed to lead. Adverse reactions are common and renal toxicity is most common (Rogan *et al*., 2001). DMSA is administered orally, more effective and less toxic. It is used in combination with EDTA for severe lead poisoning. Administration is parenteral, with adverse reactions. However, the most important factor in lead management is to reduce exposure (Rogan *et al*., 2001).

2.1.7 Lead and neurotoxicity

The most deceptive and dangerous form of lead poisoning is that affecting the nervous system. In adults, lead damage mainly causes peripheral neuropathy, which is characterised predominantly by demyelination of the nerve fibres (Needleman, 1990; Fisher and Vessey, 1998). Intense exposure to high lead levels causes encephalopathy, with the following symptoms: vertigo, insomnia, migraine, irritability and even convulsions, seizures and coma (Carpenter, 2001). Lower levels of exposure to the metal induce neuropathy, which mainly affects the developing brain and provokes behavioural and cognitive impairments (Aykin-Burns *et al.*, 2003). Epidemiological studies have shown a strong correlation between lead levels in blood and bones and poor performance in aptitude tests such as intelligence quotient or psychometric tests (Lanphear *et al.*, 2005).

A similar correlation has also been found in behavioural studies carried out on animals that had been exposed to lead immediately after birth (Aldridge *et al.*, 2005). The learning process is based on the creation and remodelling of synapses and the toxic effect of lead on this process suggests that this metal specifically damages the synaptic function. The high vulnerability of children to lead is aggravated by the fact that they are fed on formula milk prepared with water rich in lead or if they ingest flakes of lead-based paint (Needleman, 1990). Lead uptake through the blood-brain barrier and into the brain proceeds at an appreciable rate, consistent with its action as a potent central neurotoxin. The transport mechanism is not totally understood, but it most likely involves passive uptake of $PbOH^{+}$ ions (Wang *et al.*, 2006). On the other hand, astrocytes may be vulnerable to the toxic effects of Pb^{2+}. Both in astroglia cells and in neurones, lead uptake is mediated by calcium channels (Xiao *et al.*, 2006).

The effects of lead on the brain, including mental retardation and cognitive deficit, are mediated by its interference with three major neurotransmission systems: the dopaminergic, cholinergic and glutamatergic systems (Baranowska and Chlubek, 2006). The effects of lead on the first two systems are well established, but their mechanisms have not yet been described exhaustively. Glutamate binds to membrane receptors of different types. Micro molar concentration of lead can block the ionic flux through the membrane channel associated to a specific class of glutamate receptors, called N-methyl-D-aspartate (NMDA) type. Through the functioning of the associated ionic channel, the NMDA receptors play an important role in excitatory synaptic transmission and, because of some of their peculiar characteristics, these receptors appear to be involved in the processes of neural network creation and, consequently, in memory and learning functions (Toscano and Gullarte, 2005; Gardoni and Di Luca, 2006).

Lead disrupts the normal physiologic processes in the central nervous system due to the similarity of the ionized lead to calcium, as both are divalent cations (Bressler and Goldstein, 1991). Lead suppresses activity-associated Ca^{2+}-dependent release of acetylcholine, dopamine and amino acid neurotransmitters (Lasley *et al.,* 1999; Devoto *et al.,* 2001), but also increases basal release (Bressler and Goldstein, 1991). It has also been shown that lead affects pre-synaptic Ca^{2+} channels involved in neurotransmitter release (Audesirk, 1993). However, lead can disrupt the physiologic effects of calcium at concentrations lower than those of calcium. Lead also has a high affinity for calmodulin, a calcium sensor that is capable of binding and being activated by metal ions (Kursula and Majava, 2007). Lead intoxication affects the developing brain more severely (Aldridge *et al.,* 2005). Lead causes an inappropriate release of neurotransmitter at rest and competes with calcium to interfere with evoked neurotransmitter release. This increase in basal release and decrease in evoked

release may interfere with selective pruning of synaptic connections in the brain during the first years of brain development and disrupt brain plasticity (Bressler and Goldstein, 1991).

Glutamate is the most common neurotransmitter in the brain. It is always excitatory, usually due to simple receptors that increase the flow of positive ions by opening ion channels (Gardoni and Di Luca, 2006). Glutamate stimulations are terminated by a chloride-independent membrane transport system that is used only for reabsorbing glutamate and aspartate across the pre-synaptic membrane. Lead is critical for learning in the developing brain and it interferes with the glutamate, which is thought to be associated with neuronal development (Penugonda *et al.*, 2006).

The receptor NMDA is responsible for the development of brain plasticity and is blocked selectively by lead (Garza *et al.*, 2006). This disrupts long-term potentiation, which comprises the permanent retention of newly learned information. Calcium is a physiologic activator of protein kinase C (PKC) more avidly than lead, which causes further problems with neurotransmitter release (Bressler *et al.*, 1999). Impaired PKC function may also compromise the second-messenger systems within the cell, leading to changes in gene expression and protein synthesis. High blood lead levels impair endothelial cell function in the blood-brain barrier leading to haemorrhagic encephalopathy, characterised by seizures and coma (Bressler and Goldstein, 1991).

2.2 Oxidative Stress

Although oxygen metabolism is essential for life, it has been established that it results in the formation of partially reduced oxygen species (Koppenol *et al.*, 1992; Koksoy *et al.*, 2000; Trouyz, 2004, Valko *et al.*, 2007). One electron deduction of oxygen produces superoxide, whereas two electron reductions produce hydrogen peroxide. Therefore, electron flow through oxygen-utilising processes such as the mitochondrial electron transport chain,

flavoproteins, cytochrome P450 and oxidases, is tightly coupled to avoid partial reduction of oxygen (Suzuki *et al.,* 1997).

Normal cellular homeostasis is a delicate balance between the rate and magnitude of oxidant formation and the rate of oxidant elimination. Trace amounts of these powerful oxidants are involved in defence of cells against invading bacteria and other microorganisms (Troy *et al.*, 1996). However, their uncontrolled overproduction injures cells by attacking vital cellular constituents such as proteins, methylene groups of polyunsaturated fatty acids (PUFA) of cell membranes or molecules that make up the genetic material of the cell (Yamagishi *et al.*, 2001). Certain body cells (hepatocytes, neurones, glial cells, erythrocytes *etc*) are vulnerable to reactive oxygen species (ROS) because their plasma membranes contain large amounts PUFA (Alverez and Storey, 1995).

Under normal physiological states, excess ROS are continuously inactivated by endogenous antioxidants. When the rate of production of these ROS is greater than their rate of removal, oxidative stress occurs (Chulbek, 2003; Agarwal and Prabakaran, 2005).

2.2.1 Generation and effects of free radicals

The term free radicals have been equated with reactive species or oxidants. Biological reduction of molecular oxygen (O_2) generates products collectively termed ROS (Chlubek, 2003). By definition, a radical is a molecule possessing an unpaired electron such as superoxide, nitric oxide, hydroxyl, alkoxyl and alkyl-peroxyl (lipid) (Agarwal *et al.*, 2003). Many of the oxidants generated by toxins and their metabolites fall within this category of ROS, and these modify cell functions, endanger cell survival or both (Agarwal and Prabakaran, 2005).

A critical function of ROS is immunological host response. Experimental evidence has implicated ROS in the pathogenic mechanism of several diseases (Wang *et al.,* 2002). Severe oxidative stress results in necrotic cell death. Generation of reactive species during hyperoxia (breathing of >95% oxygen) or reperfusion of an ischaemic tissue leads to tissue ischaemia-reperfusion injury (Bilenko *et al*., 1989; Ayo *et al*., 1999; Olatunji-Akioye and Akinrinmade, 2006; Akinrinmade and Olatunji-Akioye, 2007) resulting in necrosis (Kuzu *et al.*, 1998; 2000). A moderate exposure to ROS can result in cell death that usually occurs 20-24 hours after the initial insult (Troy *et al.*, 1996). In most cases, delayed cell death resembles apoptosis since DNA fragmentation and other features of apoptosis bear evident. It is not clear how ROS can induce delayed cell death or apoptosis. Potential pathways, that once altered by ROS that will lead to delayed cell death include energy sources (mitochondria, activation of poly-ADP ribosyl synthase), ionic homeostasis, signal transduction and membrane structural integrity (Dawson and Dawson, 1995; Szabo *et al.*, 1996).

Oxidative stress can, however, be evaluated directly or indirectly. Direct evaluation is via the detection of circulating free radicals *in situ* through electromagnetic resonance spectroscopy (ERS) and chemiluminiscent spectroscopy (CLS) (Rimbach *et al.*, 1999). The former is based on the measurement of energy variations when an odd (unpaired) electron aligns in response to an external magnetic field (Halliwell *et al.*, 1992), while the latter measures the electromagnetic radiation of excited molecules (Lunec, 1996). Indirect methods for evaluating oxidative stress are determination of circulating antioxidant agents such as proteins, vitamins, minerals and enzymes (Koracevic *et al.*, 2001).

2.2.2 Antioxidant net

Due to the constant production of ROS and other free radicals during metabolic processes, the cell has developed a powerful, complex defence system that limits its exposure

to these agents. These are called antioxidants. Antioxidants, therefore, are molecules that prevent an unlimited generation of free radicals or inhibit their reaction with biological structures (Chaudiere and Ferrari-Iliou, 1999; Chihuailaf *et al.*, 2002). To maximize protection, plasmic and intracellular antioxidants are tuned to achieve the utmost suppression of those reactions that generate free radicals (Yu, 1994). They are distributed mostly in the cell's organelles where, due to intense activity and metabolic functions, free radical generation will be higher, both in membranes and cytosol (Larkins, 1999). Some proteins are also part of the antioxidant net, such as those in charge of storage and transport of metallic ions; they trap and limit exposure to Fe^{3+} and Cu^{2+}. An excess of these metal ions can promote free-radical generation. Proteins with antioxidant activity are ferrin, transferrin, ceruloplasmin, hepatoglobin, metallothioneine, haemopexine and carnosine (Halliwell *et al.*, 1992; Morrisey and O'Bien, 1998).

2.2.3 Classification of antioxidants

Several antioxidants are enzymes or essential nutrients or include these in their molecular structure (Machlin and Bendich, 1987). These include reduced glutathione, glutathione peroxidase, superoxide dismutase, catalase and nutrients such as ascorbic acid, α-tocopherol, uric acid, zinc etc. An essential nutrient is a compound that must be eaten because the organism is unable to synthesize it, Based on this characteristic, some authors classify antioxidants as non-enzymatic and enzymatic (Chaudiere and Ferrari-Iliou, 1999; Larkins, 1999). However, another frequently used classification is based on the protective mechanism used by the antioxidants, grouping them in those that prevent free-radical generation and those that trap free-radicals (Miller *et al.*, 1993). They are also classified according to their intracellular or extracellular location (Larkins, 1999).

2.2.3a Non-enzymatic antioxidants

Non-enzymatic antioxidants constitute a heterogeneous group of hydrophobic and hydrophilic molecules that trap free radicals and create chemical species that are less noxious to cell integrity (Halliwell and Chirico, 1993). Essentially, they give an electron to a free radical to stabilize it (Larkins, 1999). Hydrophilic non-enzymatic antioxidants are located mainly in the cytosol, mitochondrial and nuclear matrixes and in extracellular fluids. They are vitamin C, glutathione, uric acid, ergothioneine and polyphenolic flavonoids (Chihuailaf *et al.*, 2002). Another example of hydrophobic non-enzymatic antioxidant is alpha-tocopherol (vitamin E).

2.2.3b Enzymatic antioxidants

Some scientists state that the antioxidant function performed by enzymes has advantages compared to antioxidant compounds because their activity is regulated according to cellular requirements (Chihuailaf *et al.*, 2002). This can be induced, inhibited or inactivated by endogenous effectors (Harris, 1992). Evidence of the importance of antioxidant enzymes in protection against oxidant agents has been provided (Ho *et al.*, 1998). When using transgenic mice designed to over-express the activity of some antioxidant enzymes, it was noticed that there is a tolerance of certain tissues when they are exposed to toxics and pathologic conditions that promote ROS action (Chihuailaf *et al.*, 2002).

Enzymatic antioxidants catalyse electron transfer from a substrate towards free-radicals. Later, the substrates or reducing agents used in these reactions are regenerated to be used again. They achieve this by using the NADPH produced in different metabolic pathways (Chaudiere and Ferrari-Iliou, 1999). A prolonged exposure to ROS may result in diminished NADPH concentration which is needed, though some enzymatic antioxidants do not consume

co-factors. Enzymatic antioxidants include superoxide dismutases, catalase, glutathione peroxidase and glutathione reductase (Chen *et al.*, 2000; Powell, 2000; Guo *et al.*, 2003).

2.3 Vitamin C (Ascorbic acid)

2.3.1 Historical development

Scurvy, the dread of sailors past, conjures up images of perilous sea voyages and rough tattooed men laid low by ignorance of a vital amine, later designated vitamin C (which, as it happens, is not an amine). While scurvy devastated sea fearers, it was endemic in the land-bound, occurring widely wherever fruit and vegetables were in short supply (Padayatty and Levine, 2001). Military campaigns from the crusades to the Napoleonic Wars, the American Civil War, and even World War I were stymied by widespread and often fatal scurvy among the troops. The anti-scorbutic principle, identified and named ascorbic acid (vitamin C), in 1932, is a simple water-soluble sugar-like molecule (Padayatty and Levine, 2001).

Jane Lind's treatise on scurvy, first published in 1973, reported the onset of the disease in sailors after a month and a half at sea and described lassitude as its early and invariable symptom (Padayatty and Levine, 2001). Early experiments, though perhaps flawed, showed that the consumption of as little as 10 mg of vitamin C a day would prevent signs of clinical scurvy. Although minute amounts will forestall death, the optimum requirements for good health are not known (Padayatty and Levine, 2001).

2.3.2 Mechanism of antioxidant effects

Vitamin C (chemical names: ascorbic acid and ascorbate) is a six-carbon lactone which is synthesized from glucose by many animals. It is synthesized in the liver in some mammals and in the kidneys, in birds and reptiles, but not in humans and non-human

primates and guinea pigs (Padayatty *et al.*, 2003). Humans and primates lack the terminal enzyme in the biosynthetic pathway of ascorbic acid, L-gulonolactone oxidase, because the gene encoding for the enzyme has undergone substantial mutation so that no protein is produced (Nishikimi *et al.*, 1994).

Vitamin C is an electron donor and, therefore, a reducing agent. All known physiological and biochemical actions of vitamin C are due to its action as an electron donor. It donates two electrons from a double bond between the second and the third carbons of the 6-carbon molecule (Padayatty *et al.*, 2003). By donating its electrons, it prevents other compounds from being oxidized; hence vitamin C is called an antioxidant. When vitamin C donates electrons, it is itself oxidized and the electrons are lost sequentially. The species formed after the loss of one electron is a free radical, semi-dehydroascorbic acid or ascorbyl radical (Padayatty *et al.*, 2003). As compared to other free-radicals, ascorbyl radical is relatively stable with a half-life of 10^{-5} seconds and is fairly reactive. Thus ascorbate is a good free-radical scavenger due to its chemical properties (Buettner and Moseley, 1993).

2.3.3 Physiological role of vitamin C

Vitamin C is a water-soluble micronutrient required for multiple biological functions. It serves as a co-factor for several enzymes participating in the post-translational hydro-translational hydroxylation of collagen, in the biosynthesis of carnitine, in the conversion of the neurotransmitter dopamine to norepinephrine, in peptide amidation and in tyrosine metabolism (Duarte and Lunec, 2005).

In addition, vitamin C is an important regulator of iron uptake. It reduces ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions, thus promoting dietary non-heme iron absorption from the gastro-intestinal tract, and stabilizes iron-binding proteins (Arrigoni and De Tullio, 2002). Most animals are unable to synthesize vitamin C from glucose, but humans, other primates, guinea

pigs and fruit bats lack the last enzyme involved in the synthesis of vitamin C (gulonolactone oxidase) and so require the presence of the vitamin in their diet (Duarte and Lunec, 2005). Therefore, deficiency or lack of vitamin C in diet of these animals induces prolonged post-translational modification of collagen that causes scurvy and eventually cell death/apoptosis (Halliwel, 2001; Arrigoni and De Tullio, 2002).

In addition, vitamin C is a potent reducing agent and scavenger of free-radicals in biological systems (May, 1999; Duarte and Lunec, 2005). Briefly, the mono-anion form (ascorbate) is the predominant chemical species at physiological pH. Ascorbate readily undergoes two consecutive, yet reversible one-electron oxidation to generate dehydroascorbate (DHA) and an intermediate ascorbate free radical (Figure 2.3). These properties make ascorbate an efficient electron donor in many biological redox reactions, capable of replacing potentially and highly damaging radicals by the poorly reactive ascorbate radical, where $AscH^-$ represents ascorbate, $Asc^{\cdot-}$ represents the ascorbate free radical and X^* represents the oxidizing species (Figure 2.3):

$$AscH^- + X^* \longrightarrow Asc^- + XH$$

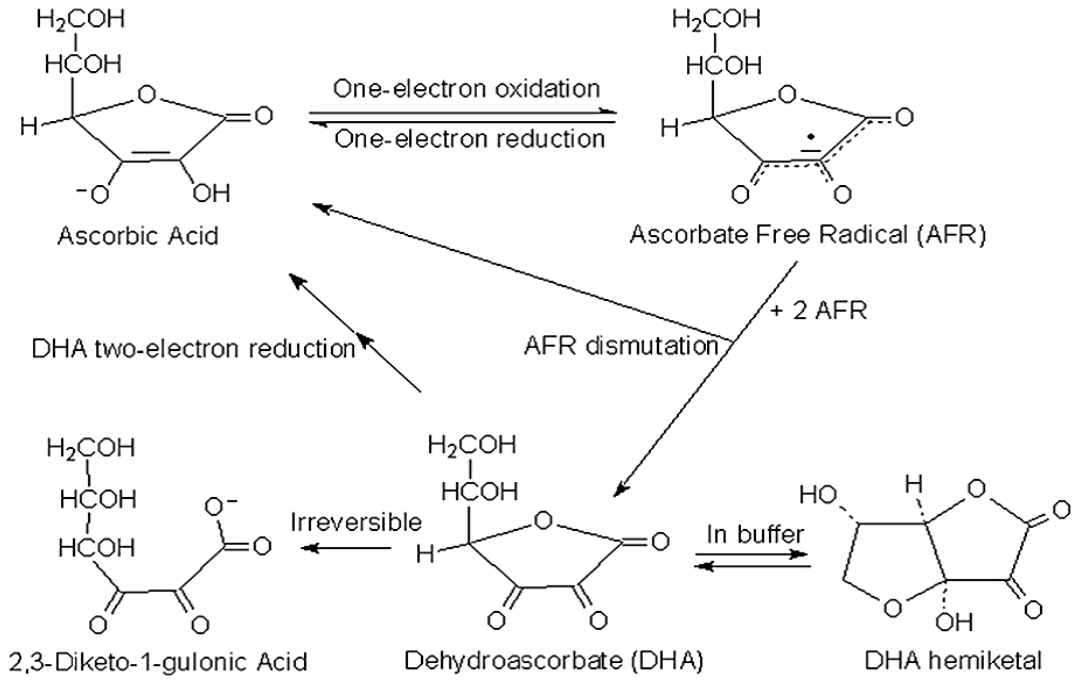


Figure 2.3. Redox metabolism of ascorbate (Harrison and May, 2009).

The one-electron oxidation of ascorbate generates the ascorbate free radical with one electron delocalized between three oxygen atoms, which on further oxidation originate dehydroascorbate (DHA). DHA is an unstable molecule and can be decomposed or reduced back to ascorbate (Harrison and May, 2009). Furthermore, vitamin C has the ability to recycle other important antioxidant molecules such as alpha-tocopherol and glutathione from their respective radical species (Duarte and Lunec, 2005). Low levels of vitamin C have been demonstrated in several conditions of increased oxidative stress such as cancer, diabetes mellitus, smoking habits and some environmental toxicosis (Pilidori *et al*., 2001; Jaruga *et al*., 2002).

It is well known that reactive oxygen species can act as sub-cellular messengers in several gene regulatory and signal transduction pathways, so antioxidants are capable of activating certain genes and signalling pathways by modulating the redox state of the cell (Allen and Tresini, 2000). In addition, the binding activity of certain transcription factors is

determined by the redox state of the cell. NFkB and AP-1 are well-known mediators of redox-responsive gene expression (Zhou *et al.*, 2001). Studies with some cultured cells have shown that vitamin C can affect gene expression and this seems to be mediated by its redox effects (Duarte and Lunec, 2005). Due to its pro-oxidant or antioxidant properties, it can modulate nuclear binding of redox sensitive transcription factors such as apoptotic factor 1 (AP-1) and NFkB (Duarte and Lunec, 2005).

Figure 2.4 below describes the proposed mechanisms of action of vitamin C. Briefly; vitamin C enters cells as ascorbate directly through sodium-dependent transporter. Alternatively, DHA generated in the extracellular space upon oxidation of ascorbate by ROS or by free metal ions is taken up through the glucose transporters; and once inside the cells, it is reduced back to vitamin C. The intracellular vitamin C enrichment and the oxidation events putatively generated during the vitamin C recycling from DHA have the potential to change the cellular redox status. The hydrogen peroxide (H_2O_2) formed extracellular during the auto-oxidation of vitamin C can modulate the binding activity of redox-sensitive transcription factors, resulting in the activation of gene expression. The end-point effects are cell type-specific and include the activation of stress response genes, cell killing by apoptosis, promotion of cell differentiation, cell proliferation and possibly DNA repair (Duarte and Lunec, 2005).

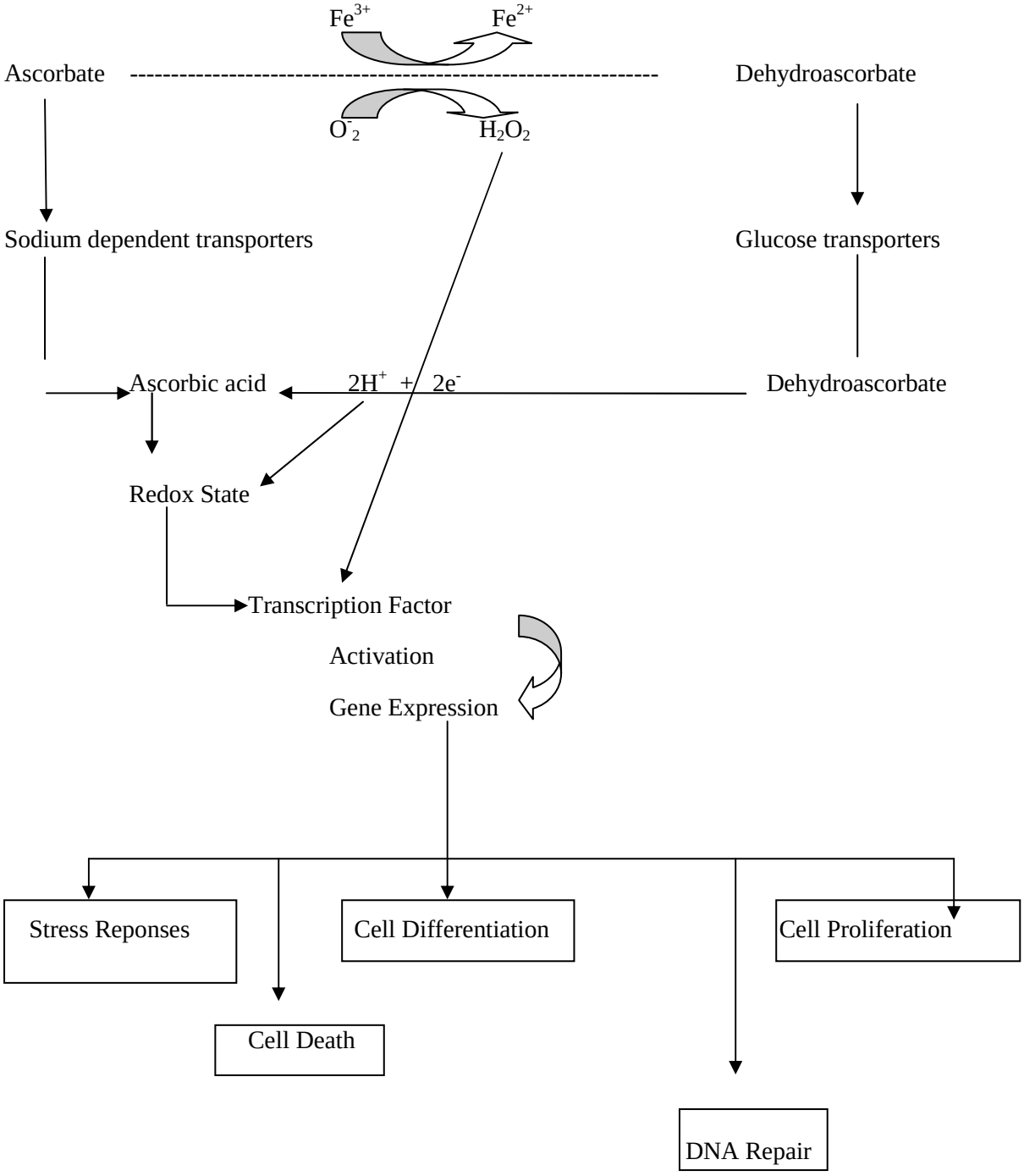


Figure 2.4: Proposed mechanisms of action of Vitamin C in Mammalian cells (Duarte and Lunec, 2005).

2.4 Lead and Oxidative Stress

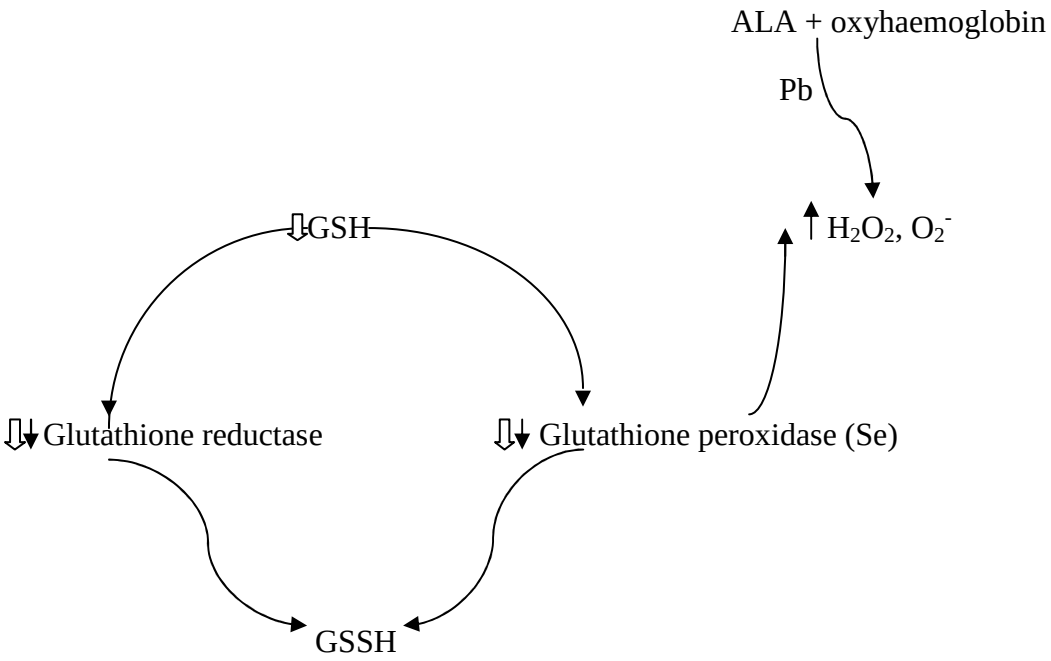
Lead is a ubiquitous element in the environment causing oxidative burst in the exposed individuals and leading to tissue damage (Flora *et al*., 2004). It is supposed that oxidative stress is one of the possible mechanisms of lead toxicities, and it is more pronounced in the young than older subjects (Tandon *et al*., 2002; Aykin-Burns *et al*., 2003). This phenomenon of lead-induced oxidative stress has been reported recently by several authors (Erdogan *et al*., 2005; Penugonda *et al*., 2006).

Lead is known to produce oxidative stress in animals, and it is manifested as an increase in lipid peroxidation in different tissues such as liver, erythrocyte, brain, muscle, *etc* (Erdogan *et al*., 2005; Faix *et al*., 2005), and subacute exposure to lead in rats has resulted in increased oxidative stress markers such as thiobarbituric reactive substances (TBARS), glutathione, reduced forms of glutathione and reduction in specific activities of membrane bound enzymes, acetylcholinesterase and monoamine oxidase (Saxena and Flora, 2004; Yedjou and Tchounwou, 2007).

It has been suggested that lead-induced oxidative stress might result from accumulation of delta-aminolevulinic acid (ALA), a potential endogenous source of free radicals (Nihei and Guilarte, 2001; Stoleski *et al*., 2008), induced by inhibition of ALA dehydratase by lead (Patrick, 2006). It can directly interact with biological membranes to induce lipid peroxidation, and can also induce decreases in the activities of free radical scavenging enzymes (Moreira *et al*., 2001; Aykin-Burns *et al*., 2003; Kasperczyk *et al*., 2004).

Glutathione and glutathione–related enzymes such as glutathione reductase and glutathione peroxidise are endogenous antioxidants that play a role in the scavenging of free-radicals. They contain sulfhydryl groups (-SH) or thiol groups. Lead has been shown to have

a high affinity for thiol groups and therefore, decreases the levels of these compounds (Gurer and Ercal, 2000) (Figure 2.5).



Key: ALA = Delta aminolevulinic acid, ⇩↓ = Reduction or elevation due to upregulation or decreased availability, ↓ = Reduction due to direct binding to Pb, GSH = Reduced glutathione, GSSG = Oxidized glutathione.

Figure 2.5: Effect of lead on glutathione metabolism (Patrick, 2006).

Lead-induced oxidative stress plays a significant role in the regression phase of hepatic hyperplasia with the generation of lipoperoxide and other oxidants, and the induced expression of cytokine mediators including tissue necrotic factor (TNF-α). These mediators are associated with the significant decline in intracellular adenosine triphosphate (ATP) concentration observed in mouse hepatocyte cultures, and in oxidative DNA damage and apoptosis of hepatocytes (Milosevic and Maier, 2000; Mudipalli, 2007).

A schematic diagram of lead-induced oxidative stress has been described below.

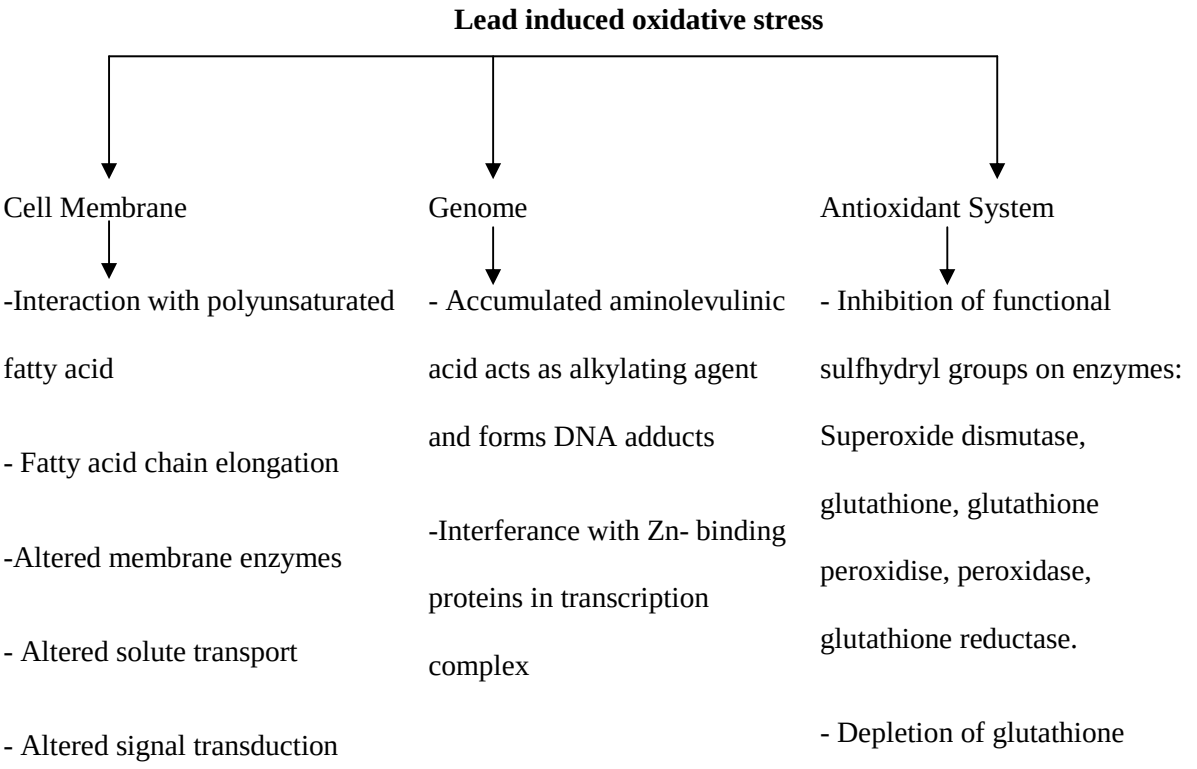


Figure 2.6: Modes of lead-mediated oxidative stress (Mudipalli, 2007)

Further evidence for lead-induced oxidative stress was found in recent investigations that buttress the fact that antioxidants were able to reverse lead-induced oxidative stress. The involvement of ROS in lead toxicity and the use of antioxidant, N-acetylcysteine in treating plumbism have been reported (Flora *et al*., 2004). It has been demonstrated that this antioxidant reversed lead-induced oxidative stress in human carcinoma cells [HepG2] (Yedjou and Tchounwou, 2007).

2.5 Nervous System and Oxidative Stress

Reactive oxygen species (ROS) are known to damage all cellular biomacramolecules (lipids, sugars, proteins and polynucleotide), and this damage can lead to the generation of

secondary products that can be deleterious to cells just as the initial ROS. The central nervous system is particularly vulnerable to oxidative insult on account of the high rate of O_2 utilization, the relatively poor concentrations of classical antioxidants and related enzymes, and the high content of polyunsaturated lipids [the biomacromolecules most susceptible to oxidation] (Sayre *et al*., 2008).

The mitochondria are the main cellular organelles involved in cellular respiration. The very high oxygen demand of the brain makes it rich in mitochondria. Mitochondrial production of ROS initially arises as superoxide anion radical from the side reaction of oxygen intercepting single electrons from the electron transport chain (Sayre *et al*., 2008). Oxidative phosphorylation in mitochondria is the major source of ROS. In addition, there are regionally high concentrations of redox-active transition metals (copper and iron) capable of the catalytic generation of ROS (Salazar e*t al*., 2006; Donnelly *et al*., 2007; Sayre *et al*., 2008). Therefore, oxidative stress is a common mechanism for neuro-related disorders, where damage to neurones can reflect both an increase in oxidative processes and a decrease in antioxidant defences.

To protect itself under normal physiological conditions, the inner membrane of mitochondria incorporates a number of free-radical scavengers and enzymatic ROS removal systems. In certain pathological states, it is apparent that mitochondrial defences can become compromised, due to either genetic mutations or an increase in radical production (Sayre *et al*., 2008). Evidence has indeed shown that mitochondrial-derived oxidative stress is a primary event associated with neuro-disorders (Maneuso *et al*., 2006). It is pertinent to consider the major chemical consequences of unchecked ROS production with respect to damage inflicted to biomacromolecules.

Oxidation of protein side-chains by ROS and reactive nitrogen species (RNS) such as peroxynitrite usually results either in introduction of hydroxyl groups or in the generation of protein-based carbonyls (Dalle-Done *et al*., 2005; Davies, 2005). A substantial fraction of protein damage that occurs under conditions of oxidative stress may represent adduction of secondary products of the oxidation of sugars, termed glycoxidation, or of the oxidation of polyunsaturated lipids, termed lipoxidation (Sayre *et al*., 2001).

Oxidative stress conditions and the occurrence of iron or copper-mediated Fento-chemistry also result in oxidative damage to nucleic acids, in particular, ribose nucleic acid (RNA). 8-Hydroxyguanosine (8-OHG) is a marker of nucleic acid oxidation commonly observed in the cytoplasm of neurones (Honda *et al*., 2005). Polyunsaturated lipids in lipoproteins and membranes are also highly susceptible to oxidative damage. The availability of a particularly weak bis-allylic C-H bond in polyunsaturated lipids allows for propagation of a free-radical chain auto-oxidation process known as lipid peroxidation, with alkylperoxy radicals as chain carriers (Sayre *et al*., 2008). If there is extensive membrane oxidation, neurotoxicity could theoretically arise in part from compromises in membrane function, affecting maintenance of membrane potential, synaptic signalling, *etc.* Such compromise has only been documented so far for mitochondria (Zhao *et al*., 2005; Sen *et al*., 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental Animals

Fifty-three (53) adult Wistar rats of both sexes (26 males, 27 females) weighing between 80-100 g were obtained from the Small Animal Experimental Unit of the Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. The animals were kept in the laboratory of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria. They were allowed to acclimatize for at least two weeks in the laboratory before the commencement of the experiment. The rats were fed on pellets made from growers' mash (Vital Feeds, Jos), maize bran and groundnut cake at a ratio of 4:2:1. (Feed proximate composition: Moisture = 7.83, crude protein = 16.03, crude fibre = 14.07, lipid = 2.05, ash = 10.52, NFE = 49.50, calcium = 2.15, phosphorus = 0.47). Feed and water were provided *ad libitum.*

3.2 Chemical Acquisition and Preparations

Lead acetate (May & Baker Ltd., Dagenham, England. Lot No. 62984) was purchased from a reputable scientific store in Zaria. It was prepared by reconstitution in distilled water to make 10 % stock solution. Ascorbic acid (Aldrich Chemical Co. Ltd., Gillingham Dorset, England. Cat. No. 25,556-4) was also obtained from the same scientific store in Zaria. Prior to its daily use, the ascorbic acid granules were weighed and a 10 % solution was prepared with distilled water. All other chemicals and reagents used for laboratory analysis were of analytical grade and obtained from reliable chemical stores.

3.3 Determination of Median Lethal Dose (LD_{50})

Thirteen (13) adult Wistar rats of both sexes were used for this phase of the study. The median lethal dose (LD_{50}) of lead acetate was evaluated using the method of Lorke (1983). Briefly, the evaluation was carried out in two stages. In the first stage, nine rats were divided into three groups of three animals each. Rats in group I were administered with lead acetate at 1000 mg/kg orally, while those in groups II and III were given 2,000 mg/kg and 3,000 mg/kg orally of the same lead acetate, respectively. The animals were observed for death over a period of 48 hours. Based on the result obtained, the second stage of the test was conducted as follows: Three rats individually caged and numbered I, II and III were administered the reconstituted lead acetate at a dose of 4,000 mg/kg, 4,500 mg/kg and 5,000 mg/kg, respectively. LD_{50} was then determined based on the dose-response (death) as described by Lorke (1983).

3.4 Sub-chronic Toxicological Study

Forty adult (40) Wistar rats of both sexes (20 males, 20 females), weighing between 90-110 g were used for this study. The rats were weighed and then divided into four groups of 10 rats each, comprising 5 males and 5 females. Rats in group I administered with equal volumes of distilled water (0.4 ml) served as the control group. Rats in group II were administered with lead acetate only (250 mg/kg) - a dose equivalent to $1/20^{th}$ of the LD_{50}. Those in group III were treated with 100 mg/kg of ascorbic acid, while the group IV animals were pre-treated with ascorbic acid at a dose of 100 mg/kg and lead acetate (250 mg/kg). The administration was done for each rat once daily for a period of six weeks, orally. During this period, signs of toxicity and death were recorded. In addition, the effect of the treatment on body weight changes in the rats was evaluated by weighing each animal weekly, using a digital weighing balance (Acculab EC-2100, Acculab Sartorius Group, Weender Landstrasse 94-108, 37075, Goettingen, Germany). Furthermore, a periodic evaluation of some

neurobehavioural parameters was conducted on the rats from each group. Also at the end of the experiment, the cognitive ability of the rats was assessed. The rats were examined based on their performances.

3.5 Evaluation of the effect of sub-chronic administration of lead acetate, ascorbic acid and lead acetate + ascorbic acid on:

3.5.1 Neurobehavioural changes

a. Open-field assessment

The open-field apparatus (Appendix XIV) as described by Zhu *et al*. (2001) was used to evaluate open-field activities of rats exposed repeatedly to all the treatments. The open-field activities were examined on each animal in all the groups prior to the commencement of the study (day 0) and subsequently on day 2, and thereafter on a weekly basis for the rest of the test period. The open-field apparatus was constructed using cardboard box (50 × 50 × 46 cm high) with a clear plexiglass on the inner surface. The floor of the box was divided into 25 equal squares. The parameters analysed in the open-field apparatus included motor activity (locomotor activity) and anxiety, evaluated based on the frequency of rearing, stretch-attend posture, urination and defaecation. All the open-field parameters were assessed at 07:00-10:00 h (GMT +1) on each examination day.

In general, motor activity was assessed by placing an animal in the box and then allowing it to walk freely for 3 minutes for familiarity with the environment. Then, the number of squares crossed with all the paws during the next two minutes was recorded. The same pattern of examination was done for the evaluation of rearing (Zhu *et al*. 2001). Soapy water followed by 90 % alcohol solution was used to clean the inner surface of the box between trials to remove the interfering odours left by the previous animal.

b. Neuromuscular coordination

The effects of lead acetate and ascorbic acid on neuromuscular coordination were evaluated based on the performance of the rats on inclined plane as described by Petrich (2006), with slight modifications. Briefly, each rat was evaluated by placing on an inclined plane apparatus made with an inclined rough wooden plank with thick foam pad at its bottom end (Appendix XV). The plank was first raised to an inclination of 35^{o}, and thereafter increased step-wise by 5^{o} until the subject could no longer stay horizontally on the plank for 3 s without sliding down. Angles were measured and marked on the apparatus before hand, and the angle was obtained by propping the plank on a bar with several notches that caught on a vertical bar. The test was performed with the head of the rat facing left and then right hand side of the experimenter. The highest angle at which each rat stayed and stood horizontally and facing each direction was recorded. Two trails were performed for each testing period (Petrich, 2006). This procedure was carried out on each animal in all the groups on day 0, weeks 2, 4 and 6 of the study.

c. Level of excitability

This neurobehavioural parameter was evaluated by assessing the sensorimotor reflex using the excitability score as described by Maria *et al*. (2004) and Ayo *et al*., (2006) as modified by Ambali (2009). Briefly, each rat was held by the tail upside down and held in that position for 60 seconds (Appendix XVI). Response of each rat was then scored using an ordinal scale of 1-5 as follows:

Grade 1- The wriggling of rat was low and also showed feeble fore-paw movement.

Grade 2- Rat responded through a stronger wriggling and fore-paw movement.

Grade 3- Rat vigorously wriggled and exhibited a strong fore- and hind-limb movement.

Grade 4- In addition to the observations in grade 3 above, rat made unsuccessful attempt to climb on its tail.

Grade 5- In addition to the observations in grade 3 above, rat successfully climbed the tip of its tail.

d. Learning

The effect of lead on learning task in rats and the possible ameliorative effect of antioxidant vitamin (ascorbic acid) were assessed 48 hours to the termination of the study using the step-down inhibitory avoidance learning task as described by Zhu *et al*. (2001). The apparatus (Appendix XVII) used for the learning test was a 40 × 25 × 25 cm acrylic chamber, consisting of a floor made of parallel 2-mm calibre stainless steel bars spaced 1cm apart. An electric shock was administered through the floor bars. A 2.5-cm-high, 8 × 25 cm wooden platform was placed on the left extreme of the chamber. Each animal was gently placed on the platform. Upon stepping down, the rat immediately received a single 80-volt foot-shock. If the animal did not return to the platform, the foot-shock was repeated every 5 seconds. A rat was considered to have learned the avoidance task, if it remained on the platform for more than 2 minutes. The number of foot-shocks applied before the animal learned the avoidance task was recorded as an index of learning acquisition.

e. Short-term memory

Short-term memory was assessed in individual rat from each group using the step-down avoidance inhibitory task as described by Zhu *et al*. (2001). The apparatus (Appendix XVII) used for the memory test consisted of 40 × 25 × 25 cm acrylic chamber with floor made of parallel 2-mm-calibre stainless steel bars spaced 1 cm apart. A 2.5-cm-high, 8 × 25 cm wooden platform was placed on the left extreme of the chamber. In this case, the rat was again placed gently on the platform 24 hours after performing the learning task. The time an

animal remained on the platform was recorded as an index of memory retention. Staying on the platform for 2 minutes was counted as maximum memory retention (ceiling response).

3.5.2 Haematology

At the end of the test period, rats from each group were sacrificed by decapitation after light ether anaesthesia. Blood (2.5 ml) was then collected into sample bottles containing ethylenediaminetetraacetic acid (EDTA) at the ratio of 5:1. Another blood sample (2.5 ml) was collected from the rats into test-tubes and allowed to stand for 60 minutes. The test-tubes were then centrifuged at 1000 × g for 10 minutes. Thereafter, the serum was collected from each test-tube into a clean sample tubes, which was subsequently used for the evaluation of serum biochemical parameters. The haematological parameters analysed were packed cell volume (PCV), erythrocyte count, haemoglobin concentration and absolute and differential leucocyte counts. The PCV, erythrocyte count, and the absolute and differential leucocyte counts were taken using the method described by Dacie and Lewis (1991). The haemoglobin concentration was determined using the cyanmethaemoglobin method as described by Baker and Silverton (1985).

a. Packed cell volume

Heparinised capillary tubes (John Poulten Limited, England) were inserted into the blood-containing sample bottles, leaving about 15 mm of the tubes unfilled. The open end of each of the tubes was then carefully sealed in a fine flame. The tubes were then loaded onto a micro-haematocrit centrifuge (Hawksley, England) and centrifuged at 1000 × g for 10 minutes. The PCV value for each of the tube was read using the haematocrit reader (Hawksley, England).

b. Haemoglobin concentration

20 µL of blood obtained from each rat was drawn using a pipette into a tube and then diluted with 5 ml of modified Drabkin's fluid and allowed to stand for at least 3 minutes. The absorbance of the mixture was read using a spectrophotometer (Beckman Coulter, Model BU520, Austria) at a wavelength of 540 nm against reagent blank. The haemoglobin concentration was determined using the following formula:

$$Hb = T \times C \times D/A \times 1000 \text{ (g/dL)} \quad (1)$$

Where T is the test absorbance, A is the standard absorbance, C is the concentration of cyanmethaemoglobin standard (mg/dL), while D is the dilution factor (1:250); 1000 converts the Hb concentration from mg/dl to g/dL.

c. Total erythrocyte count

0.5 ml of blood was sucked into erythrocyte pipette and then diluted with 100 ml of RBC diluting fluid (1:200). The counting was done in a Neubaeur counting chamber under a light microscope at × 40 magnification.

d. Absolute and differential leucocyte count

A drop of blood was placed on one end of a glass slide (UNISCOPE, England) and, using a spreader, a blood film was made and allowed to air-dry. Leishman stain was then poured to cover the film for 2 minutes. The film was then rinsed in buffered distilled water and allowed to stand for 10 minutes. The film was later viewed under a microscope in an oil immersion at × 1,000 magnification.

e. *In vitro* erythrocyte osmotic fragility

Osmotic fragility of the erythrocytes *in vitro* was determined by measuring the release of haemoglobin from blood added to test-tubes containing serially diluted phosphate-buffered

saline using the modified method of Oyewale *et al*. (1991). In summary, a 1 % solution was made from a 10 % buffered sodium chloride (NaCl) stock solution. A set of six test-tubes was used and each tube contained 5 ml of buffered NaCl solution with concentrations of 0.9, 0.7, 0.5, 0.3, 0.1 and 0.0, respectively. 20 µL of blood obtained from each animal was carefully added to each labelled tube. One set of the six test tubes was used to analyze each blood sample.

The tubes were gently mixed by inversion five times. They were allowed to stand for 30 minutes at room temperature and then centrifuged at 2000 × g for 10 minutes. 3.5 ml of the supernatant was aspirated into a glass cuvette and the optical density was read at the wavelength of 540 nm using a spectrophotometer (Spectronic-20, Phillip Harris Limited, Shenstone, England) with distilled water as a blank. The percent (%) haemolysis was calculated according to the equation of Faulkner and King (1970), as follows:

OD of test/OD of standard × 100 = percent (%) haemolysis, (2)

Where OD = optical density. OD of distilled water served as the standard.

A cumulative osmotic fragility curve; that is, the fragiligram was obtained by plotting percent (%) haemolysis against the saline concentrations (Oyewale *et al.,* 1991).

3.5.3 Serum biochemical tests

a. AST, ALT, LDH, CK, total protein, albumin and globulin

The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and creatine kinase (CK), and total protein and albumin concentration were determined in the serum using an autoanalyzer (Bayer Express Plus, Bayer, Germany, S/No: 15950). The serum globulin level was obtained by subtracting the albumin from the total protein value.

b. Alkaline phosphatase

The levels of serum alkaline phosphatase was measured using the method described by King and Armstrong (1934). The principle of the reaction is based on the fact that phenol released by enzymatic hydrolysis from phenol phosphate at pH 10.0 reacts with alkaline oxidizing agent, 4-aminophenozone to give a red colour, the intensity of which is proportionate to enzymatic activity.

c. Urea

Urea was analyzed using the method of Natelson *et* al. (1951), modified by Marsh *et al*. (1965). The principle is based on the fact that when urea is heated in strongly acidic conditions with substances such as diacetyl $CH_3COCOCH_{3,}$ containing two adjacent carbonyl groups or its monoxime, yellow condensation compounds are formed. The reaction is intensified by the presence of polyvalent ions such as ferric ions, and then red compound is formed. The intensity of the red complex is proportional to the concentration of urea in the sample.

d. Creatinine

Serum creatinine was analysed by the Jaffe's reaction (Miller and Miller, 1951). The principle is based on the fact that creatinine gives a red colour with alkaline solution of picric acid. The red colour is measured in the presence of a high background (yellow colour). The intensity of the colour formed is proportional to the concentration of the creatinine.

e. Uric acid

Uric acid was determined by the method of Caraway (1955). The principle is that phosphotungstic acid is reduced by uric acid in the presence of sodium carbonate to give a blue complex, which is measured colorimetrically to give a blue complex.

f. Malondialdehyde concentration

The level of thiobarbituric acid reactive substance, malondialdehyde (MDA) as an index of lipid peroxidation was evaluated in the brain sample using the double-heating method of Draper and Hadley (1990) as modified by Yavuz *et al*. (2004). The principle of the method is based on spectrophotometric measurement of the colour developed during reaction of thiobarbituric acid (TBA) with MDA. Briefly, the sample tissues from each animal was weighed and homogenized in a known volume of ice-cold phosphate buffer to obtain a 10 % homogenate, which was centrifuged at 6,000 × g for 10 minutes to obtain the supernatant. The supernatant was used to assess the level of protein and MDA in the sample. The assessment of MDA concentration in the supernatant was evaluated, thus: 2.5 ml of 100 g/L trichloroacetic solution was added to 0.5 ml supernatant of the sample homogenates and placed in a boiling water-bath for 15 minutes. The mixture was then centrifuged at 1000 × g for 10 minutes. 2 ml of the resultant supernatant was added to 1 ml of 6.7 g/L TBA in a test-tube and placed in a boiling water (100°C) bath for 15 minutes. The solutions were then cooled under tap water and the absorbance measured using a UV/visible spectrophotometer (Ultrospec 4000, Pharmacia Biotech) at 532 nm. The concentration of MDA in the tissue sample was calculated by the absorbance coefficient of MDA-TBA complex- 1.56 × 10^5/cm/M and expressed as nmol/mg of protein. The protein content of the supernatant was determined using the Biuret method.

3.5.4 Hormonal assay

Thyroid function was assessed in the test groups by measuring the concentration of total serum triiodothyronine (T_3), thyroxine (T_4) and thyrotrophin (TSH) using diagnostic kits (Fortress Diagnostics Limited, Unit 2C, Antrim Technology Park, Antrim BT41 1QS, UK; Revised October, 2007). The principle of the T_3 and T_4 tests is based on a competitive enzyme immunoassay. The essential reagents required for a solid-phase enzyme immunoassay include immobilised antibody, enzyme-antigen conjugate and native antigen. Upon mixing immobilised antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction results between the native antigen and the enzyme antigen conjugate for a limited number of insolubilized binding sites. After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by aspiration. The enzyme activity in the antibody-bound fraction is inversely proportional to the native antigen concentration. The T_3 test system procedure has a sensitivity of 0.04 ng/ml, while the T_4 procedure has a sensitivity of 100 ρg, which is equivalent to a sample containing a concentration of 0.4 µg/dl.

The essential reagents used for the TSH assay included high affinity and specificity antibodies (enzyme conjugated and immobilised), with different and distinct epitope recognition, in excess and native antigen. In this procedure, the immobilization took place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-TSH antibody. Upon mixing the monoclonal biotinylated antibody, the enzyme-labelled antibody and a serum containing the native antigen, a reaction resulted between the native antigen and the antibodies without competition or steric hindrance to form a soluble sandwich complex. Simultaneously, the complex was deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. After equilibrium was attained, the antibody-bound

fraction was separated from unbound antigen by decantation. The enzyme activity in the antibody-bound fraction was directly proportional to the native antigen concentration. The Fortress TSH microplate Elisa test system had a sensitivity of 0.078 µIU/ml for one-hour incubation and 0.027 µIU/ml for two-hour incubation.

a. Total serum triiodothyronine concentration

Before the assay was carried out, all reagents, serum references and controls (provided in the kit) were brought to room temperature (20-27°C). Briefly, 50 µl of the appropriate serum reference, control or specimen was drawn into a pipette in the assigned well. Thereafter, 100 µl of a working reagent A, T_3-enzyme conjugate solution were added to all the wells. The micro plate was then swirled gently for about 20-30 seconds to aid mixing and incubated for 60 minutes at room temperature. The content of the microplate was decanted by aspiration. 300 µl of wash buffer was added, decanted by aspiration and another 300 µl added. This process of washing was repeated thrice. A working signal reagent solution of about 100 µl was added to all the wells and incubated at room temperature for 15 minutes. 50 µl of a stop solution was added to each well and gently mixed for 15-20 seconds. The absorbance (Beckman Coulter Spectrophotometer, DU 520 UV/visible) in each well was read at the wavelength of 450 nm (using a reference wavelength of 620-630 nm to minimize well imperfections) in a microplate reader.

b. Total serum thyroxine concentration

Before proceeding with this assay, the microplate wells for each serum reference control and specimen to be assayed were formatted in duplicate. Briefly, 25 µl of the appropriate serum reference, control or specimen was pipette into the assigned well. Thereafter, 100 µl of working reagent A, T_4-enzyme conjugate solution was added to all the wells. The micro-plate was then incubated for 60 minutes at room temperature. The contents

of the micro-plate were discarded by aspiration, using a vacuum aspirator. 300 µl of wash buffer was added to the wells, aspirated and the wash buffer added again. This washing process was repeated two more times. 100 µl of working substrate solution was added to all the wells and incubated at room temperature for 15 minutes. After this, 50 µl of stop solution was added to each well and gently mixed for 15-20 seconds. The absorbance (Beckman Coulter Spectrophotometer, DU 520 UV/visible) in each well was read at the wavelength of 450 nm (using a reference wavelength of 620-630 nm to minimize well imperfections) in a microplate reader. The results were read within 30 minutes of adding the stop solution.

c. Serum thyrotropin (thyroid stimulating hormone) concentration

Before proceeding with this assay, all reagents, serum references and controls were brought to room temperature (20-27°C). The test procedure for TSH involved pipetting 50 µl of the appropriate serum reference, control or specimen into the assigned well. 100 µl of the TSH enzyme reagent was added to each well. The microplate was swirled gently for 20-30 seconds to mix, and then incubated for 2 hours at room temperature. The contents of the microplate were then discarded by decantation, by tapping and blotting of the plate dry with an adsorbent paper. About 300 µl of wash buffer was added to the wells, then decanted (tap and blot). This washing was repeated two more times. After washing, 100 µl of working substrate solution was added to all the wells, and incubated at room temperature for 15 minutes. The reaction was stopped by adding 50 µl of a stop solution to each well and mixed gently for 15-20 seconds. The absorbance (Beckman Coulter Spectrophotometer, DU 520 UV/visible) in each well was read at the wavelength of 450 nm (using a reference wavelength of 620-630 nm to minimize well imperfections) in a microplate reader. The absorbance was read within 30 minutes of adding the stop solution.

3.5.5 Gross and histopathologic examination

Each of the experimental animals was examined for visible lesions in the various organs (brain, liver, kidney and thyroid gland) and any lesion observed was recorded. Thereafter, about 2 g of the individual tissue was dissected out and placed in sample bottles containing 10 % formalin. The tissues were processed histologically using haematoxylin and eosin (H & E) staining (Luna, 1960). Tissue thickness was set at 8 µm. The slides were prepared and examined under a light microscope at different magnifications (× 40, × 100 and × 400) for any tissue changes. Pictures of the slides were taken after optical focus using a digital camera (Casio®, EX-Z80, 8.1 MP, S/N 44315714B, China).

3.6 Statistical analysis

The cumulative means were obtained by summation of values obtained during the individual study period and computing the average. Values were obtained as mean ± SEM. One-way analysis of variance (ANOVA) with Duncan's multiple range comparison post-hoc tests using the SAS$^{(R)}$ system software (San Diego, California, USA) was used to compare the differences between the test groups and those in the control treated with only distilled water. Values of $P < 0.05$ were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Mean Lethal Dose (LD_{50})

All the three Wistar rats from each of the three groups dosed at the phase I with lead acetate at 1000 mg/kg, 2000 mg/kg and 3000 mg/kg survived. The result obtained in phase II from the three groups of one animal each is presented in Table 4.1. Clinical signs observed include: weakness, inappetance, gasping, weight loss (after 24 hours), depression, rapid breathing and death.

Based on the method of Lorke (1983), the result of the LD_{50} = 4500 + 5500/2 = 10000/2 = 5000 mg/kg (Table 4.1)

4.2 Sub-chronic Toxicological Studies

4.2.1 Clinical signs

There were no apparent clinical signs observed in the control animals and those treated with lead acetate, ascorbic acid, and lead + ascorbic acid.

Table 4.1: Summary of results from phase II median lethal dose (LD_{50}) evaluation

Group	Dose (mg/kg)	No. dead/No. dosed
1	4 000	0/1
2	4 500	0/1
3	5 500	1/1

4.2.2 Effect of sub-chronic administration of lead, ascorbic acid and lead + ascorbic acid on body weight

The effect of the various treatments on body weight gain of the animals is presented in Figure 4.1. There was no significant ($P > 0.05$) difference in the cumulative weight gain of Wistar rats across the treatment groups.

4.2.3 Effect of sub-chronic administration of lead, ascorbic acid and lead + ascorbic acid on neuro-behavioural changes

a. Locomotor activity

The general pattern of the locomotor activity of the animals exposed to the different treatment groups is shown in Figure 4.2. There was a progressive decrease in the number of squares crossed by the Wistar rats in all the groups. At the end of the examination (week 6), the ascorbic acid-treated group (4.38 ± 2.55) had the highest number of squares crossed compared to values obtained in the lead-treated (2.60 ± 1.42), lead + ascorbic acid (1.29 + 1.13) and the control (1.10 + 0.90) groups. These values were, however, not significantly ($P > 0.05$) different from one another. The mean values of cumulative locomotor activity across the groups were also not different ($P > 0.05$). The lead-treated group (7.27 ± 1.08) had higher number of squares crossed than the control group (6.65 ± 2.11). The lead + ascorbic acid-treated group (3.62 ± 1.39) crossed lesser number of squares than the control (6.65 ± 2.11), lead (7.27 ± 1.08) and ascorbic acid-treated (8.31 ± 3.03) groups. Thus, rats treated with ascorbic acid (8.31 ± 3.03) crossed the highest number of squares (Figure 4.3).

b. Frequency of rearing

As presented in Figure 4.4, the general pattern of the mean frequency of rearing across the treatment groups was that of a progressive decrease throughout the study period. On day 2 of the period, the mean frequency of rearing obtained in the lead-treated rats (10.90 ± 3.14) was significantly ($P < 0.05$) higher than that recorded in lead + ascorbic acid-treated

rats (3.20 ± 1.36). At week 1, there was no significant ($P > 0.05$) difference in the mean frequency of rearing across the groups. However at week 2, the ascorbic acid-treated group (7.56 ± 2.33) had a significantly ($P < 0.05$) higher mean frequency of rearing than those of the control (2.60 ± 1.06), lead (2.30 ± 0.83) and lead + ascorbic acid-treated (2.70 ± 1.51) groups. At week 3, there was no significant ($P > 0.05$) difference in the frequency of rearing between the groups. However at week 6, a significantly ($P < 0.05$) higher increase was observed in the mean frequency of rearing of the ascorbic acid-treated rats (2.50 ± 0.87) than that of the lead-treated rats (0.70 ± 0.21). Although the lead-treated group (0.70 ± 0.21) had a lower mean frequency of rearing than the control (1.40 ± 0.52) and the lead + ascorbic acid-treated groups (1.14 ± 0.40), this decrease was not significant ($P > 0.05$). Although there was no significant ($P > 0.05$) difference in the cumulative frequency of rearing between the groups, a lower decrease was observed in the lead-treated group (5.00 ± 0.74) than those of the control (5.52 ± 1.44) and ascorbic acid-treated (6.83 ± 1.74) groups. The cumulative frequency of rearing in the lead + ascorbic acid group (4.72 ± 0.91) was lower than the value obtained in the lead-treated group (5.00 ± 0.74) (Figure 4.5), but the difference in the values was insignificant ($P > 0.05$).

c. Stretch-attend postures

A decrease in the number of stretch-attend postures was observed in all the groups throughout the experimental period (Figure 4.6). The cumulative frequency (0.33 ± 0.08) of stretch-attend postures in the lead + ascorbic acid-treated group decreased ($P < 0.05$) significantly compared with the lead-treated group (0.82 ± 0.18). The lead-treated group (0.82 ± 0.18), however, had a higher ($P < 0.05$) cumulative frequency of stretch-attend postures than the control (0.71 ± 0.14) and those of the ascorbic acid-treated (0.67 ± 0.13) groups (Figure 4.7).

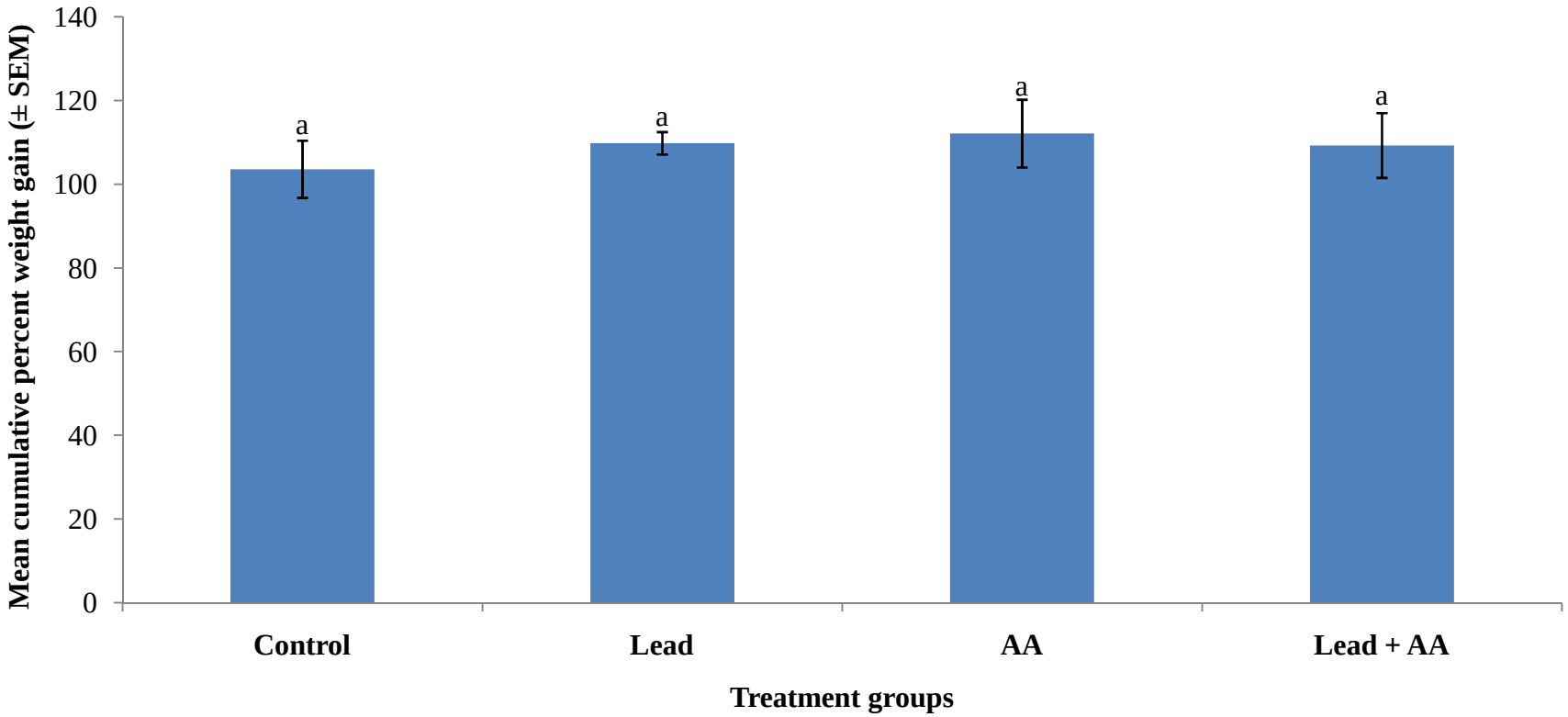


Figure 4.1: Effect of sub-chronic administration of low-dose lead acetate on body weight of control (n=10) and experimental (n=10) Wistar rats. AA = Ascorbic acid. [a] = Values with the same superscript alphabets are not significantly (P > 0.05) different.

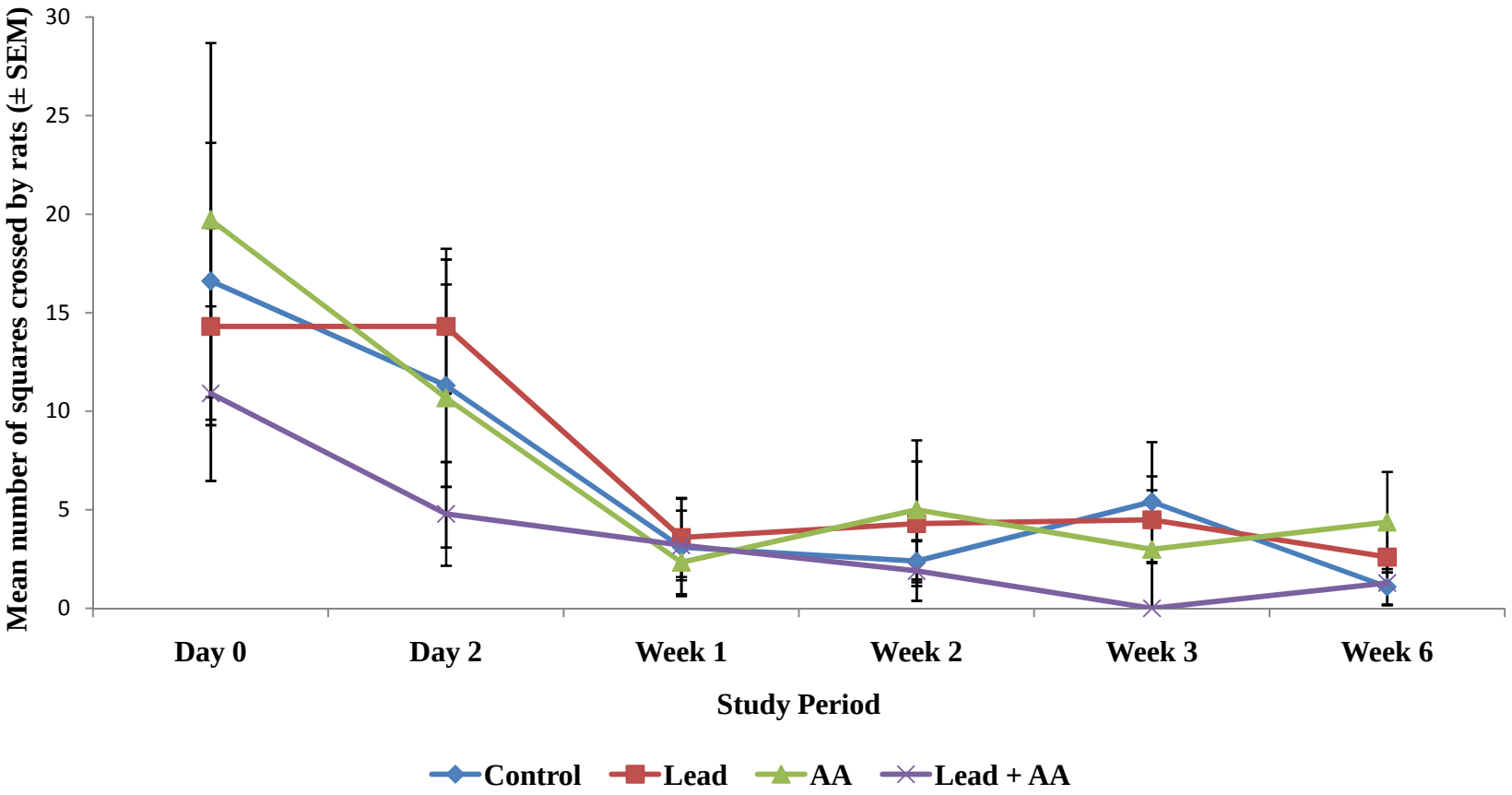


Figure 4.2: Changes in locomotor activity following exposure to repeated low-dose lead in Wistar rats. n = 10, AA = Ascorbic acid.

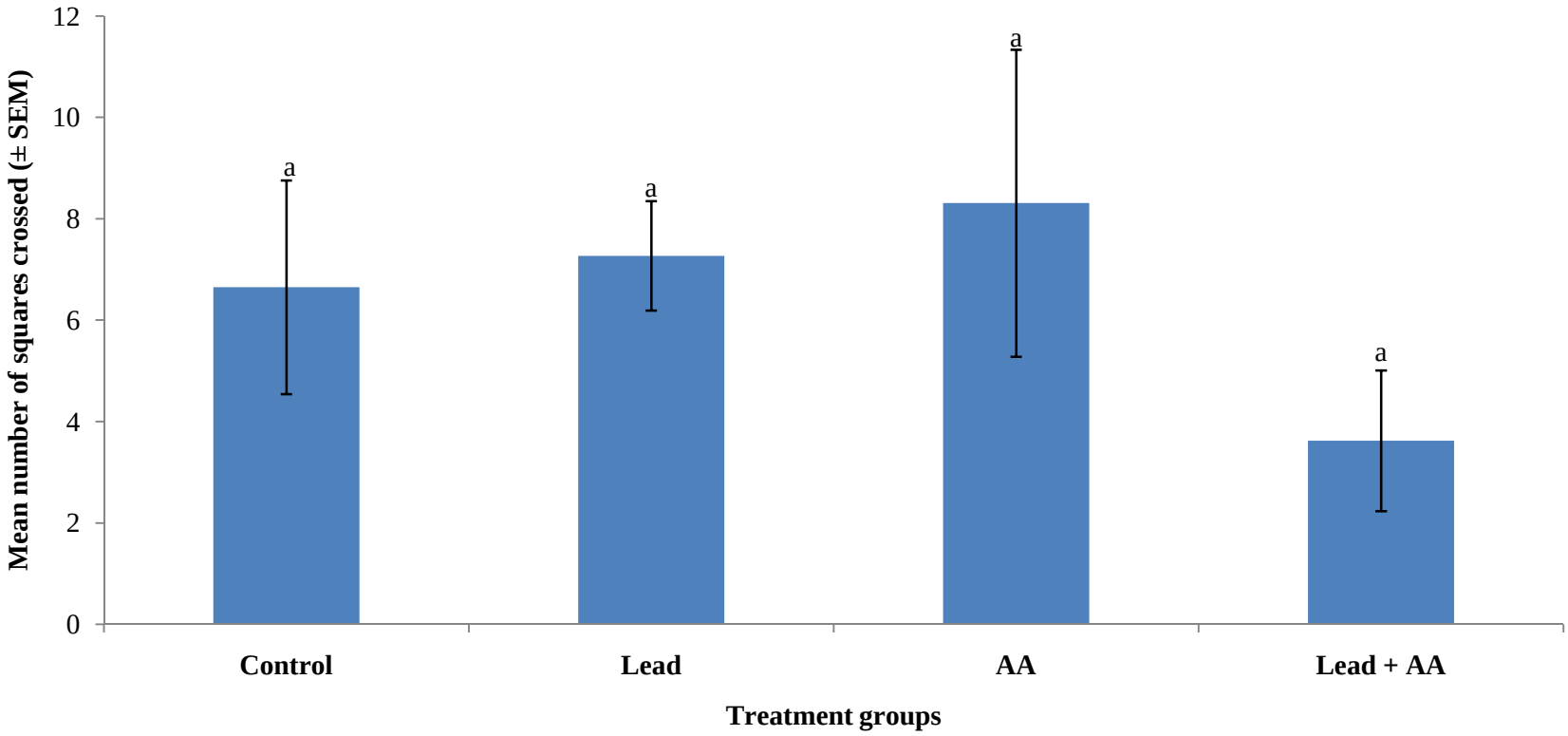


Figure 4.3: Changes in the mean cumulative locomotor activity of Wistar rats exposed to repeated low-dose lead and treated with ascorbic acid. n = 10, AA = Ascorbic acid. [a] = Values with the same superscript alphabet are not significantly (P > 0.05) different.

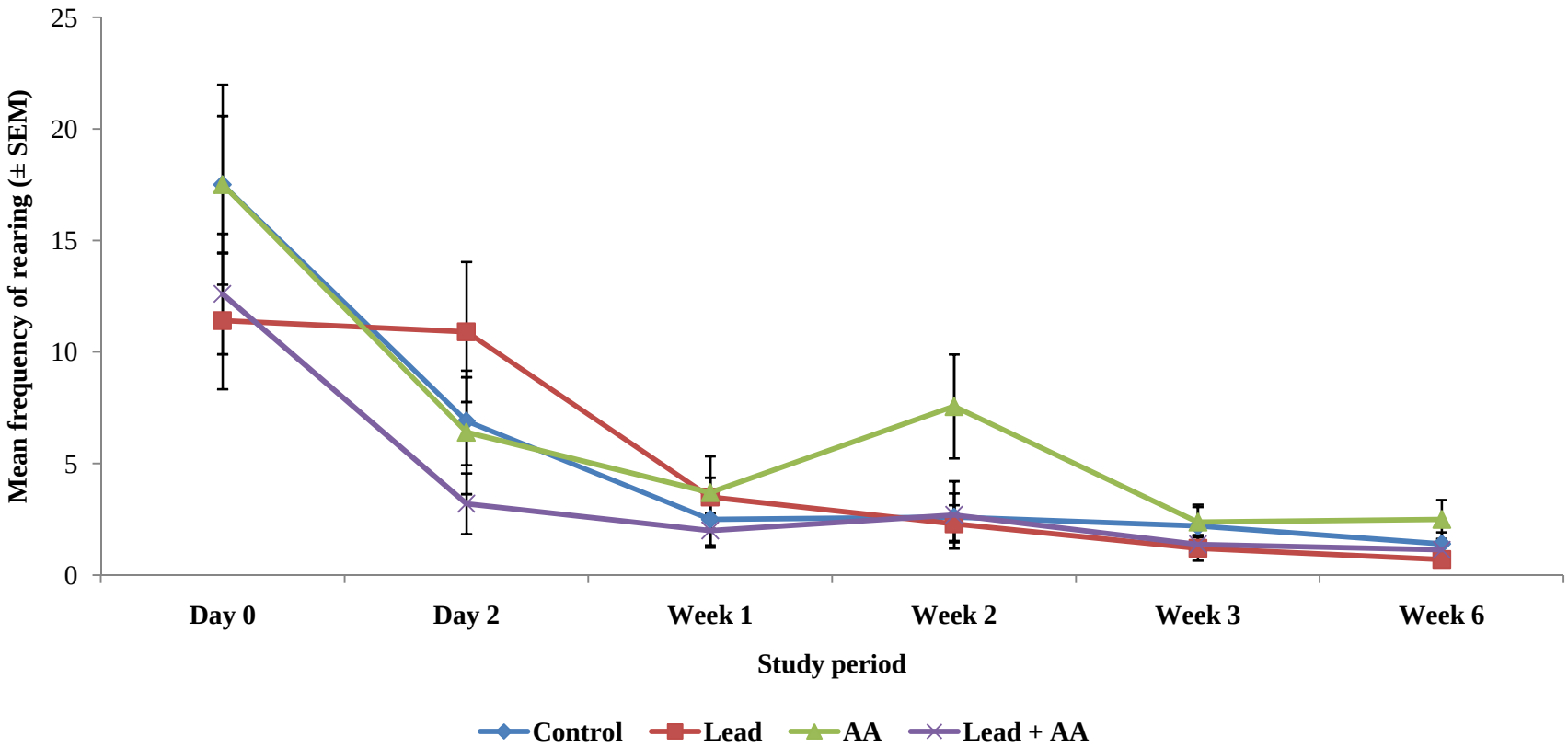


Figure 4.4: Mean changes in the frequency of rearing in Wistar rats repeatedly exposed to low-dose lead and treated with ascorbic acid. n = 10, AA = Ascorbic acid.

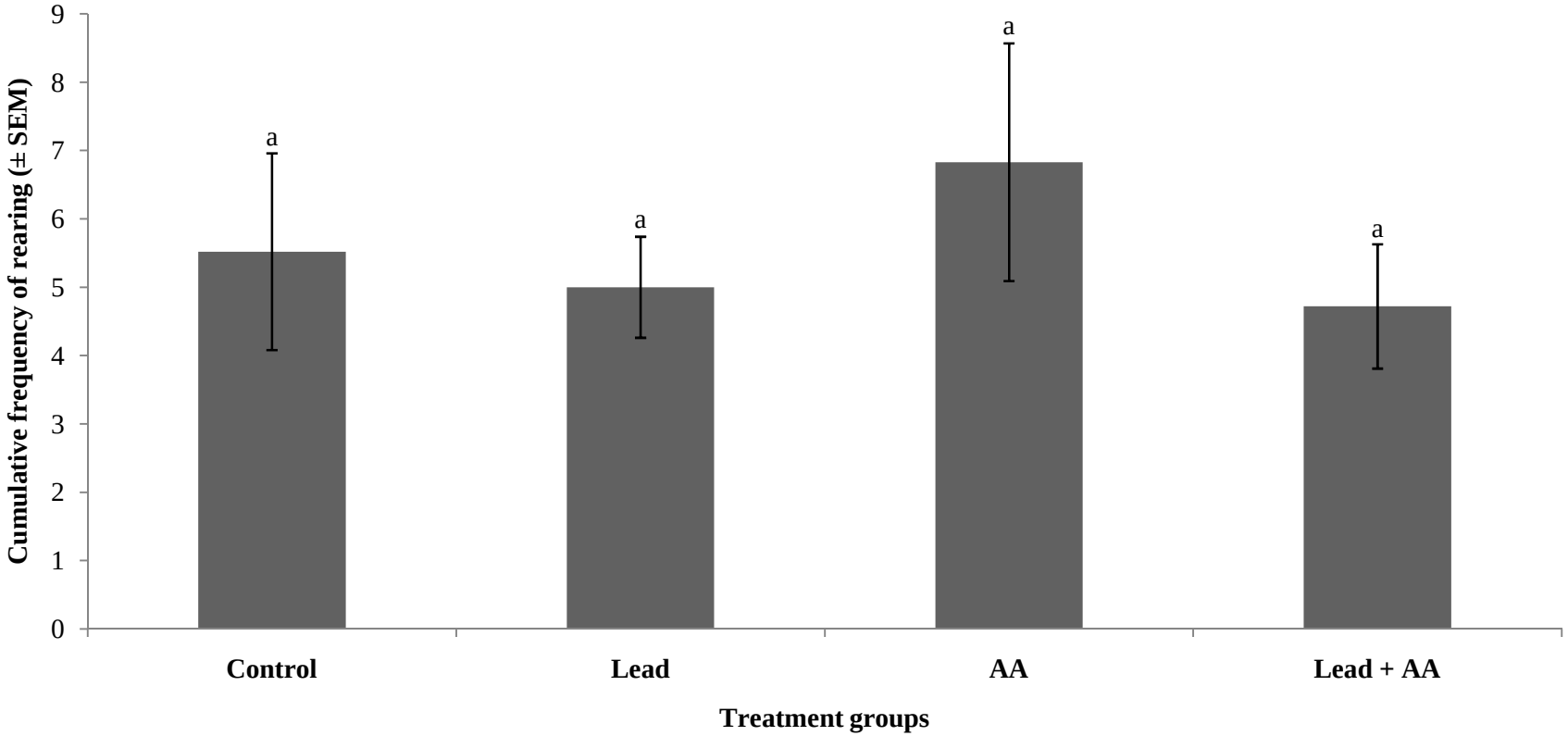


Figure 4.5: Changes in the mean cumulative frequency of rearing in Wistar rats following exposure to low-dose lead and treatment with ascorbic acid. [a] = Values with the same superscript alphabet are not significantly (P > 0.05) different. n = 10, AA = Ascorbic acid.

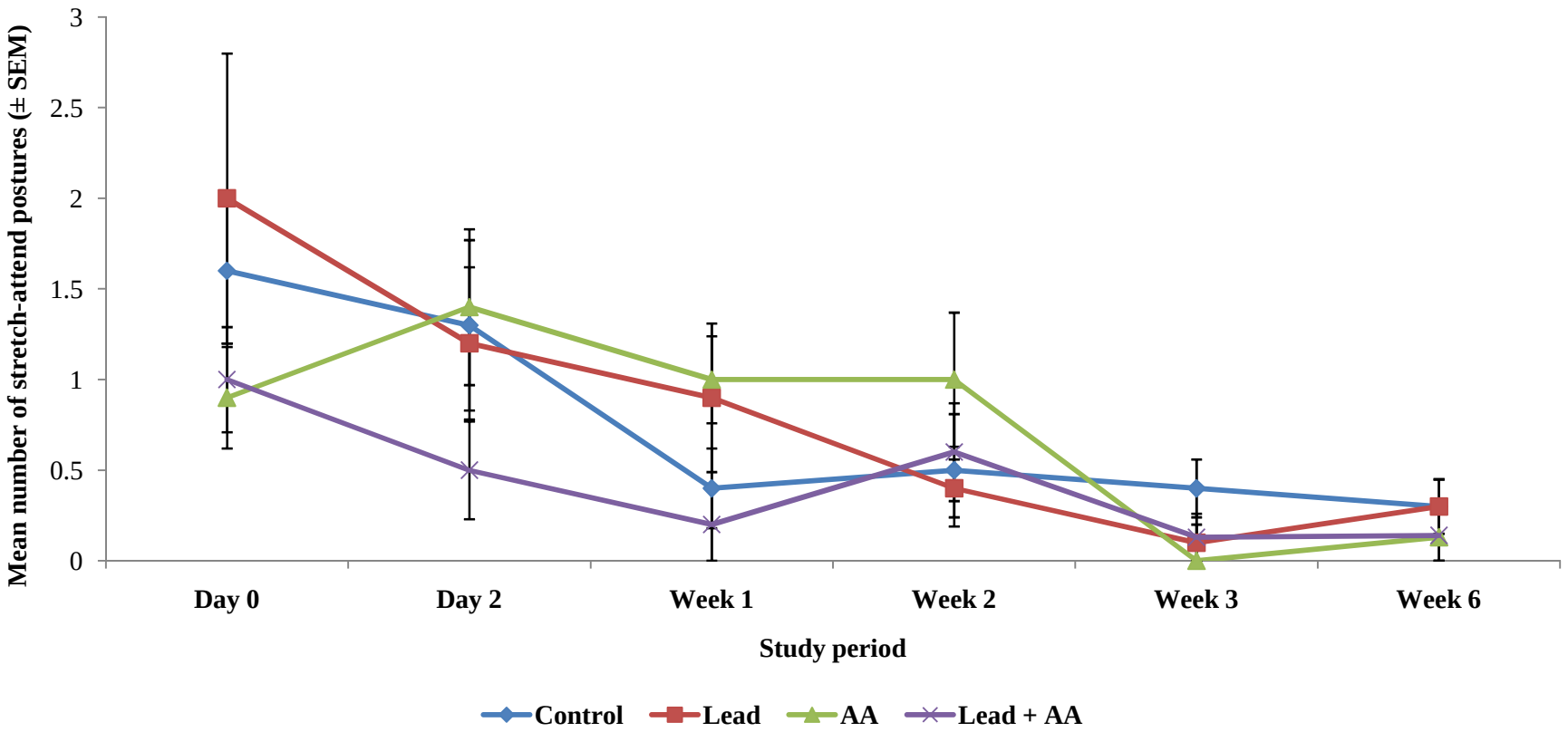


Figure 4.6: Changes in the number of stretch-attend postures in Wistar rats repeatedly exposed to low-dose lead and treated with ascorbic acid. n = 10, AA = Ascorbic acid.

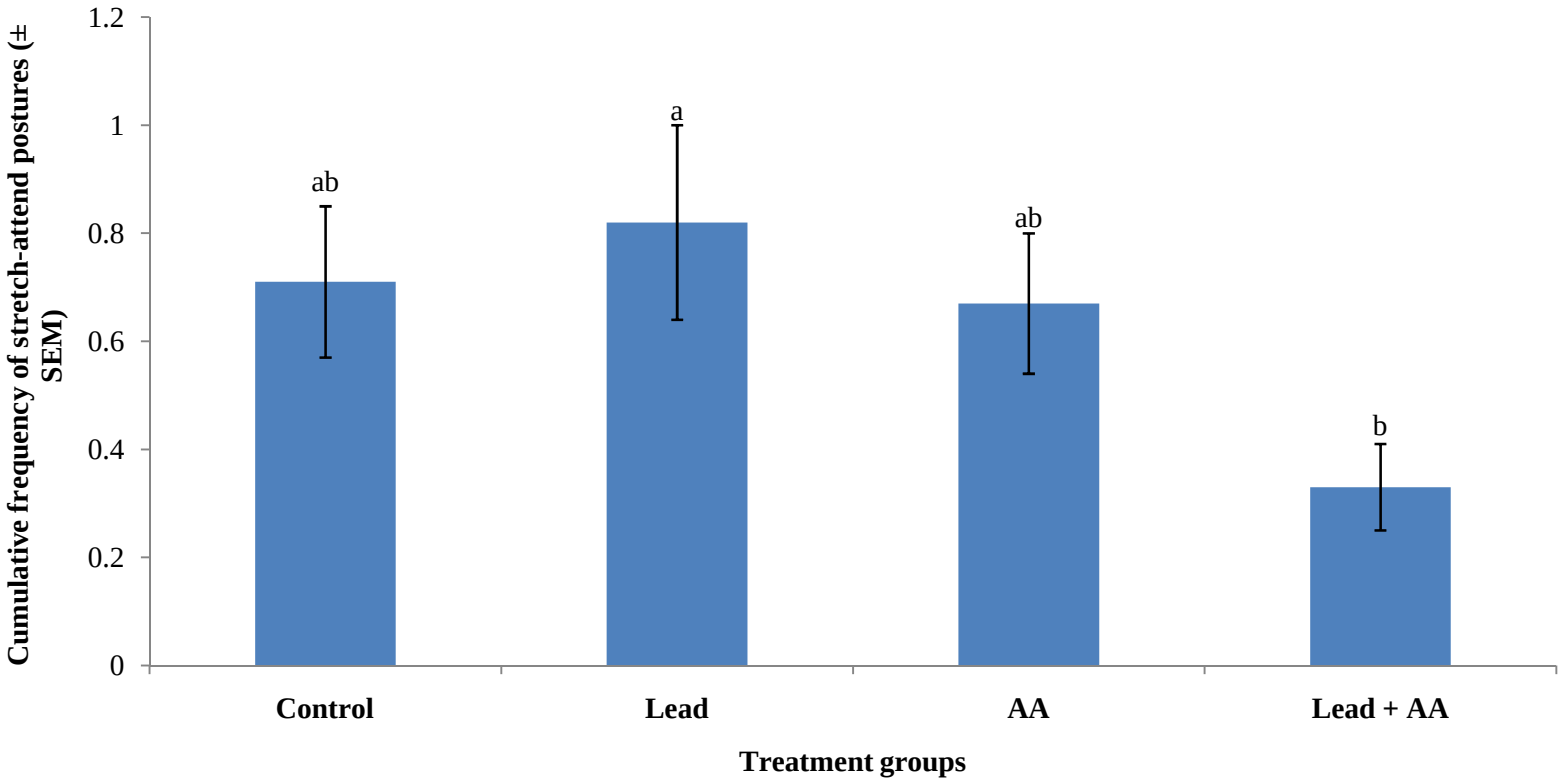


Figure 4.7: Changes in the mean cumulative number of stretch-attend postures of control (n = 10) and experimental (n = 10) Wistar rats following exposure to repeated low-dose lead and treatment with ascorbic acid. n = 10, AA = Ascorbic acid. [a, b] = Values with different superscript alphabets are significantly (P < 0.05) different.

d. Defaecation

The Wistar rats in all the experimental groups showed an increased pattern of defaecation from day 2 of the examination to week 1 (Figure 4.8). At week 1, the increases in the mean frequency of defaecation in the lead-treated group (4.50 ± 0.52) and the lead + ascorbic acid group (5.10 ± 0.85) were higher ($P < 0.05$) than that recorded in ascorbic acid-treated group (2.20 ± 0.51). The increases were, however, not significantly ($P > 0.05$) different from that of the control group (4.10 ± 0.75). Although a similar pattern was observed at week 2, all the values were lower than those obtained in week 1. The values recorded at weeks 3 and 6 were not significantly ($P > 0.05$) different from one another. The cumulative frequency of defaecation in the lead-treated group (4.02 ± 0.24) was higher than that of the control group (3.22 ± 0.37). Rats in the ascorbic acid group (2.60 ± 0.41) showed a significantly ($P < 0.05$) lower cumulative frequency of defaecation than those in the lead (4.02 ± 0.24) and lead + ascorbic acid-treated (4.43 ± 0.54) groups. A slight increase in the cumulative frequency was observed in the lead + ascorbic acid group (4.43 ± 0.54), compared with the lead-treated group (4.02 ± 0.24) (Figure 4.9).

e. Urination

The general pattern of changes in the frequency of urination in the experimental animals is presented in Figure 4.10. There was no significant ($P > 0.05$) difference in the mean frequency of urination in all the treatment groups on days 0 and 2 of the experimental period. At week 1, the frequency of urination in the lead + ascorbic acid-treated group (1.30 ± 0.34) was significantly ($P < 0.05$) higher than those of the control (0.40 ± 0.22), lead (0.50 ± 0.17) and ascorbic acid (0.50 ± 0.17) groups. The frequency of urination was not significantly ($P > 0.05$) different at weeks 2, 3 and 6 in all the treatment groups. The lead-treated rats (0.47 ± 0.12) had a higher cumulative frequency of urination than rats in the control (0.37 ± 0.12) and the ascorbic acid-treated (0.35 ± 0.09) groups. However, rats treated with lead + ascorbic

acid (0.55 ± 0.07) had a higher cumulative frequency than the lead-treated rats (0.47 ± 0.12) (Figure 4.11).

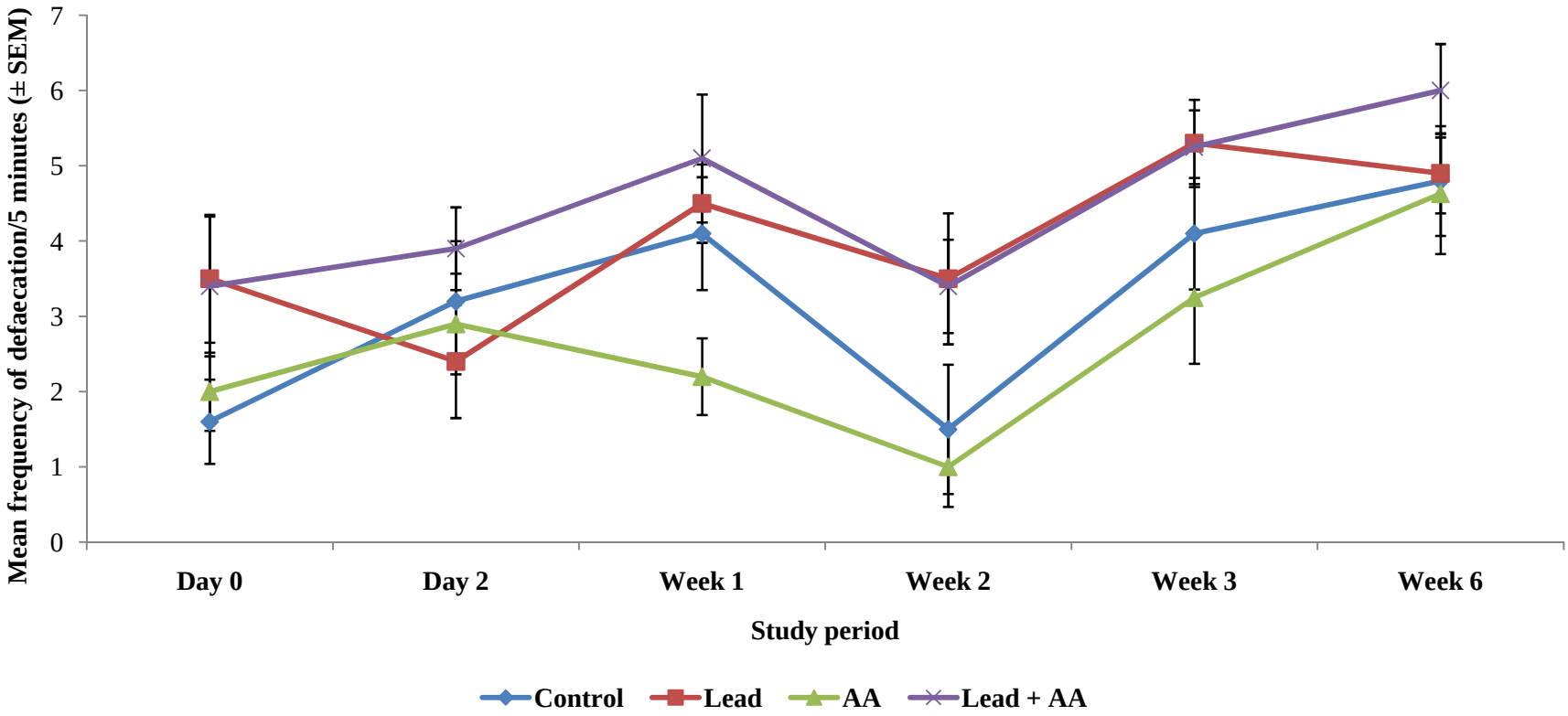


Figure 4.8: Effect of repeated exposure to low-dose lead on the frequency of defaecation in Wistar rats treated with ascorbic acid. n = 10, AA = Ascorbic acid.

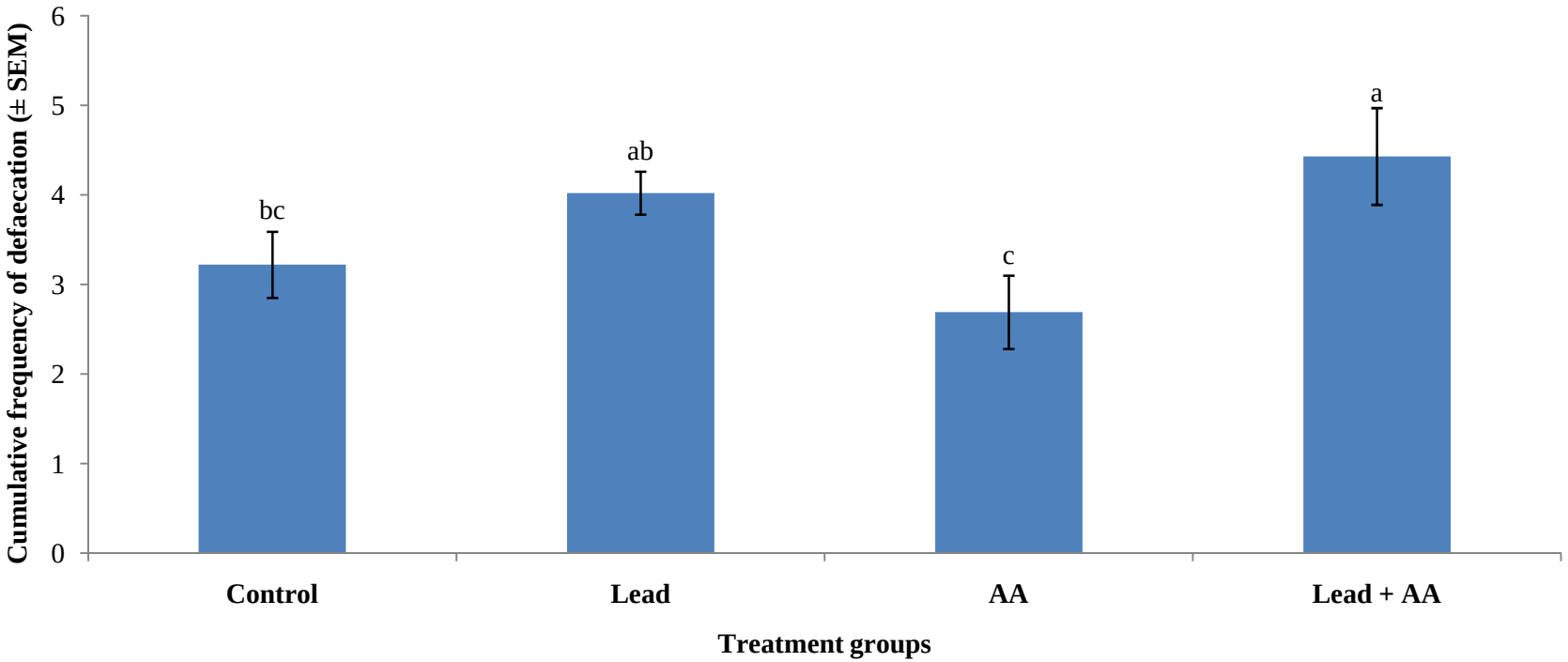


Figure 4.9: Changes in the mean cumulative frequency of defaecation in Wistar rats following exposure to low-dose lead and treatment with ascorbic acid. [a, b, c] = Values with different superscript alphabets are significantly (P < 0.05) different. n = 10, AA = Ascorbic acid.

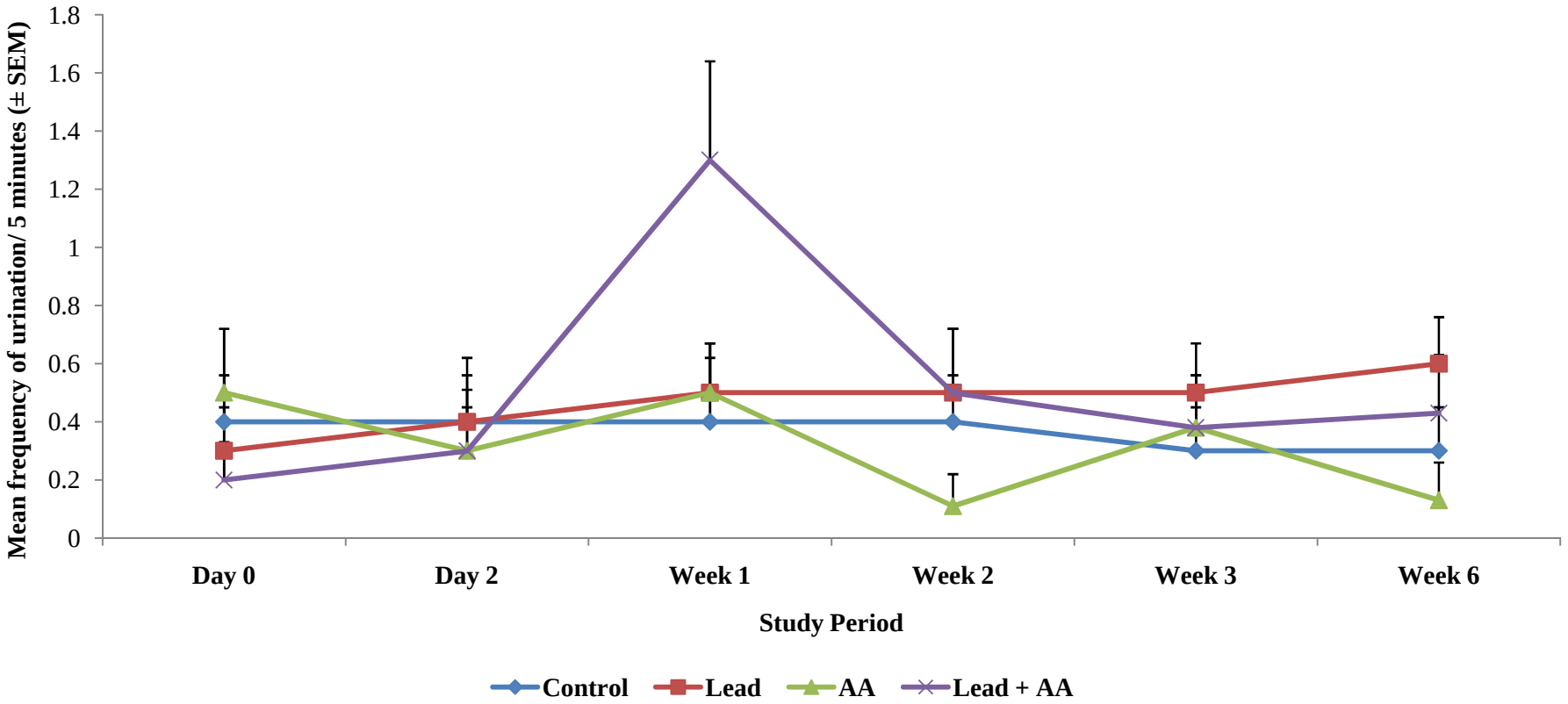


Figure 4.10: Effect of repeated exposure to low-dose lead on the frequency of urination in Wistar rats treated with ascorbic acid. n = 10, AA = Ascorbic acid.

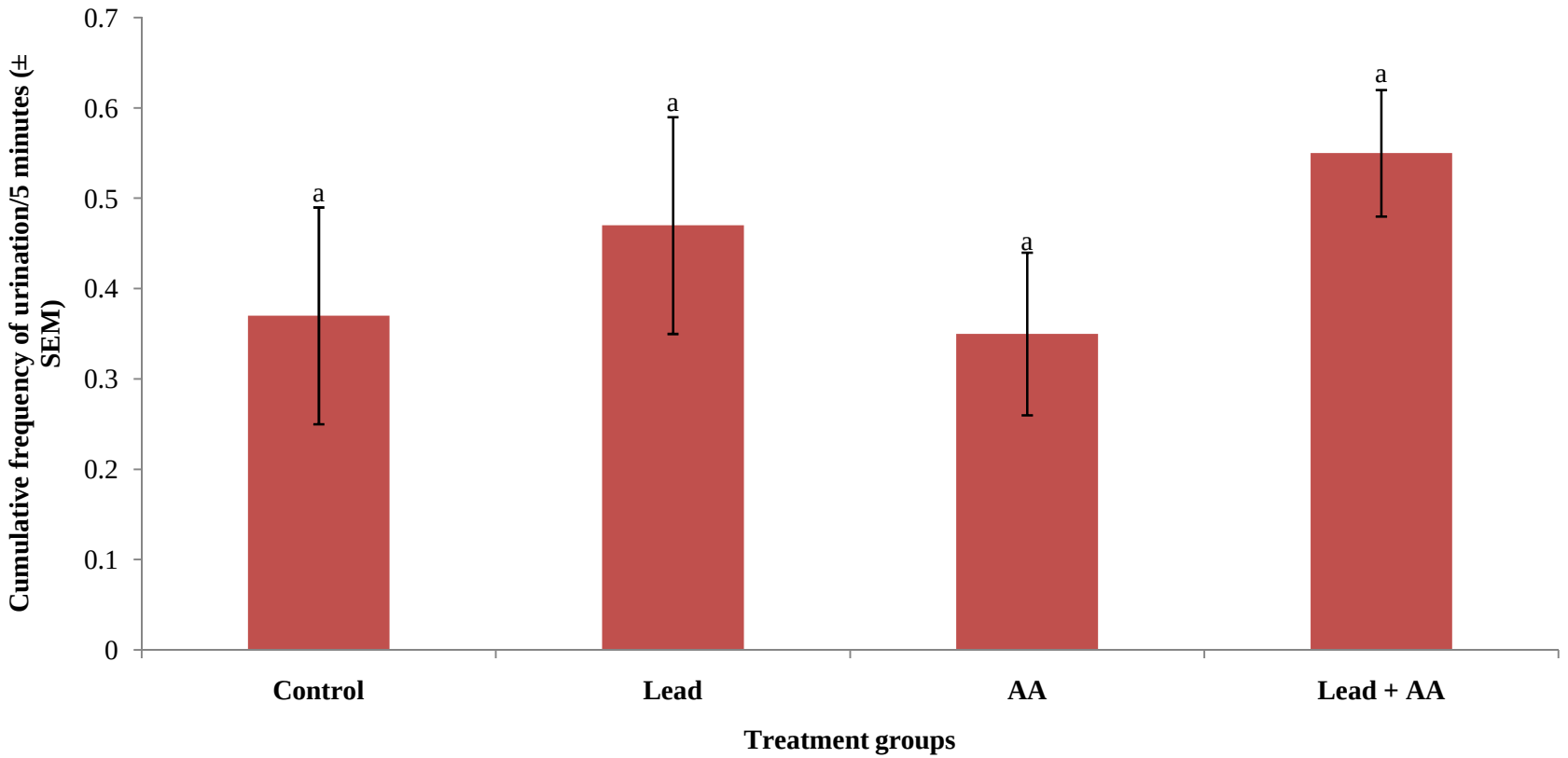


Figure 4.11: Changes in the mean cumulative frequency of urination in Wistar rats following exposure to low-dose lead and treatment with ascorbic acid. [a] = Values with the same superscript alphabet are not significantly (P > 0.05) different. n = 10, AA = Ascorbic acid.

f. Neuromuscular co-ordination

There was a steady increase in the inclined plane performance of Wistar rats in the treatment groups throughout the duration of the examination (Figure 4.12). At week 1, animals in the ascorbic acid (52.00 ± 0.82°) and the lead + ascorbic acid-treated (52.50 ± 0.83°) groups had a significantly ($P < 0.05$) lower angle of inclination than the lead-treated (55.00 ± 1.29°) and control (55.00 ± 1.67°) groups. At week 2, a significant ($P < 0.05$) decrease was obtained in the inclined angle of the control group (54.00 ± 1.25°) compared with those of the lead (57.50 ± 2.01°), ascorbic acid (56.00 ± 1.25°) and the lead + ascorbic acid-treated groups (56.50 ± 1.07°). No significant ($P > 0.05$) difference between the treatment groups was observed at week 3. However, at week 4, rats in the lead-treated group (54.00 ± 2.21°) had a significantly ($P < 0.05$) lower inclined plane performance than those in the lead + ascorbic acid-treated group (55.50 ± 0.90°). There was a lower cumulative inclined plane performance in the lead-treated group (56.50 ± 1.10°) compared with that of the control animals (57.00 ± 0.73°), but the difference was not significant ($P > 0.05$). Also at week 4, the lead + ascorbic acid-treated group (55.13 ± 0.54°) had a lower inclined performance compared with the lead-treated group (56.50 ± 1.10°). On the overall, there was no significant ($P > 0.05$) difference in the inclined plane performance of Wistar rats across the groups (Figure 4.13).

g. Level of excitability

The excitability of the experimental animals progressively increased with the duration of the experiment (Figure 4.14). There was no significant ($P > 0.05$) difference in the excitability scores of Wistar rats in all the treatment groups from day 2 of the study period to week 6. The difference in the cumulative excitability scores between the groups was also not significant ($P > 0.05$) (Figure 4.15).

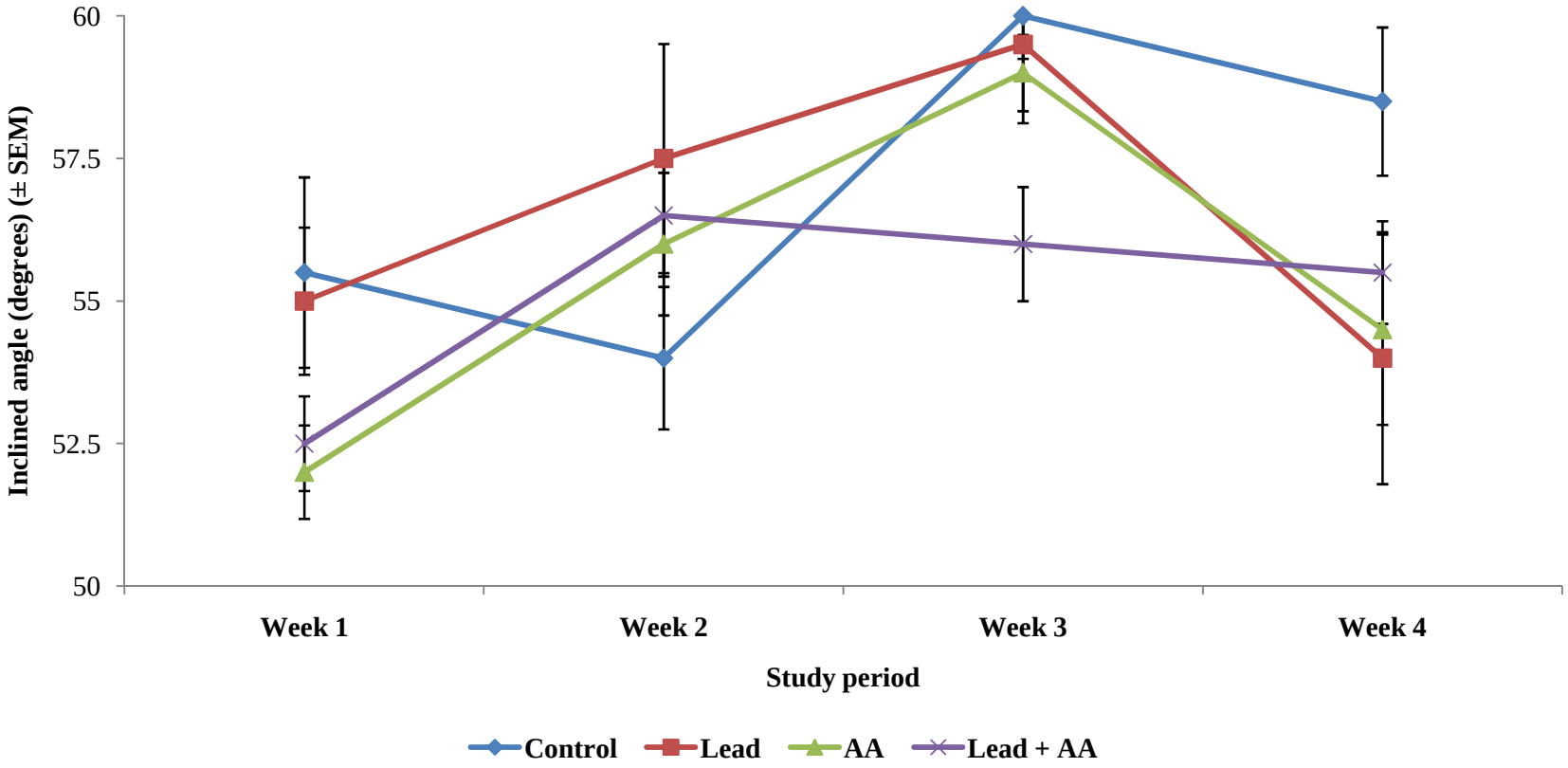


Figure 4.12: Mean changes in the neuromuscular coordination of rats repeatedly exposed to low-dose lead and treatment with ascorbic acid. n = 10, AA = Ascorbic acid.

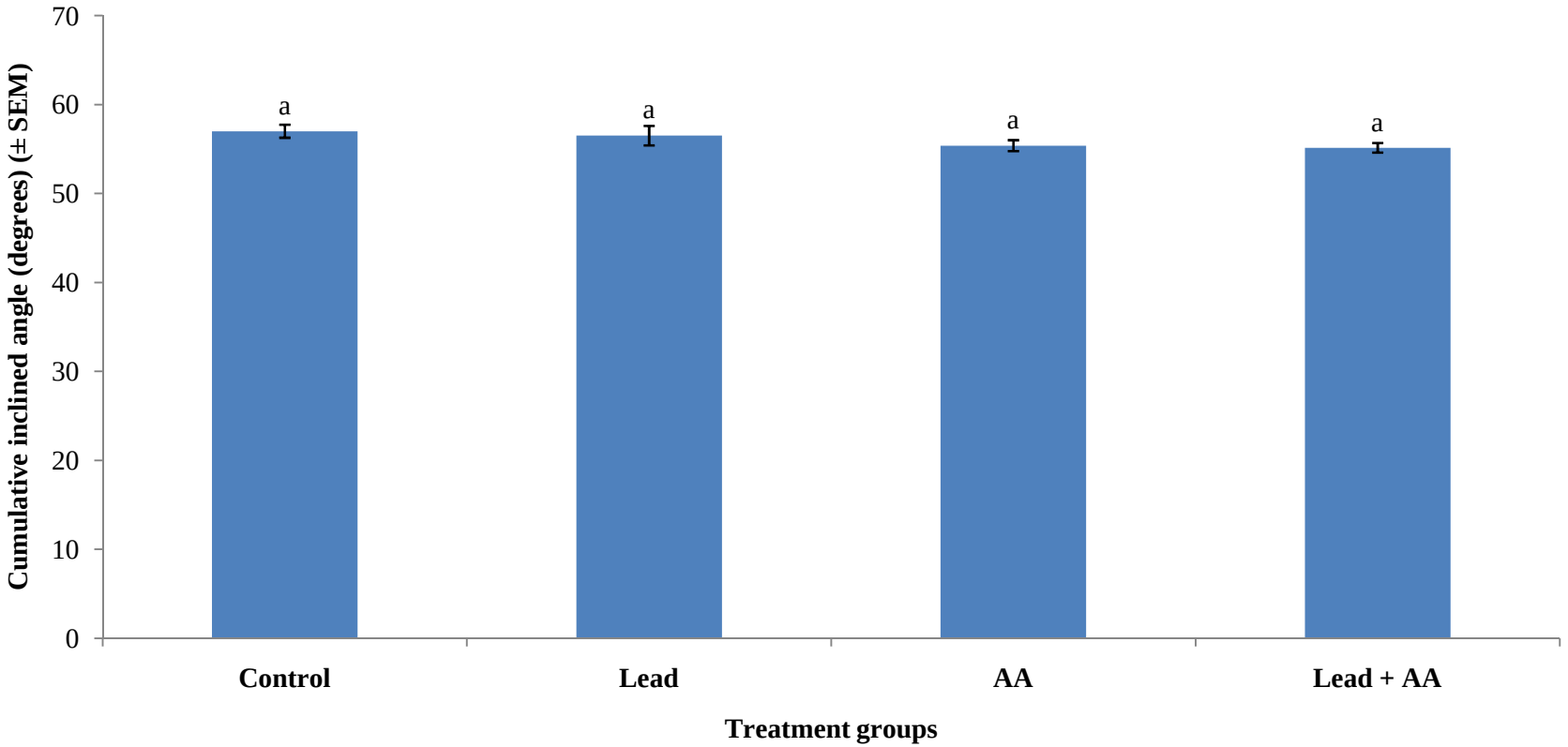


Figure 4.13: Mean cumulative changes in the inclined plane performance of Wistar rats following repeated exposure to low-dose lead and treatment with ascorbic acid. n = 10, AA = Ascorbic acid. [a] = Values with the same superscript alphabets are not significantly (P > 0.05) different.

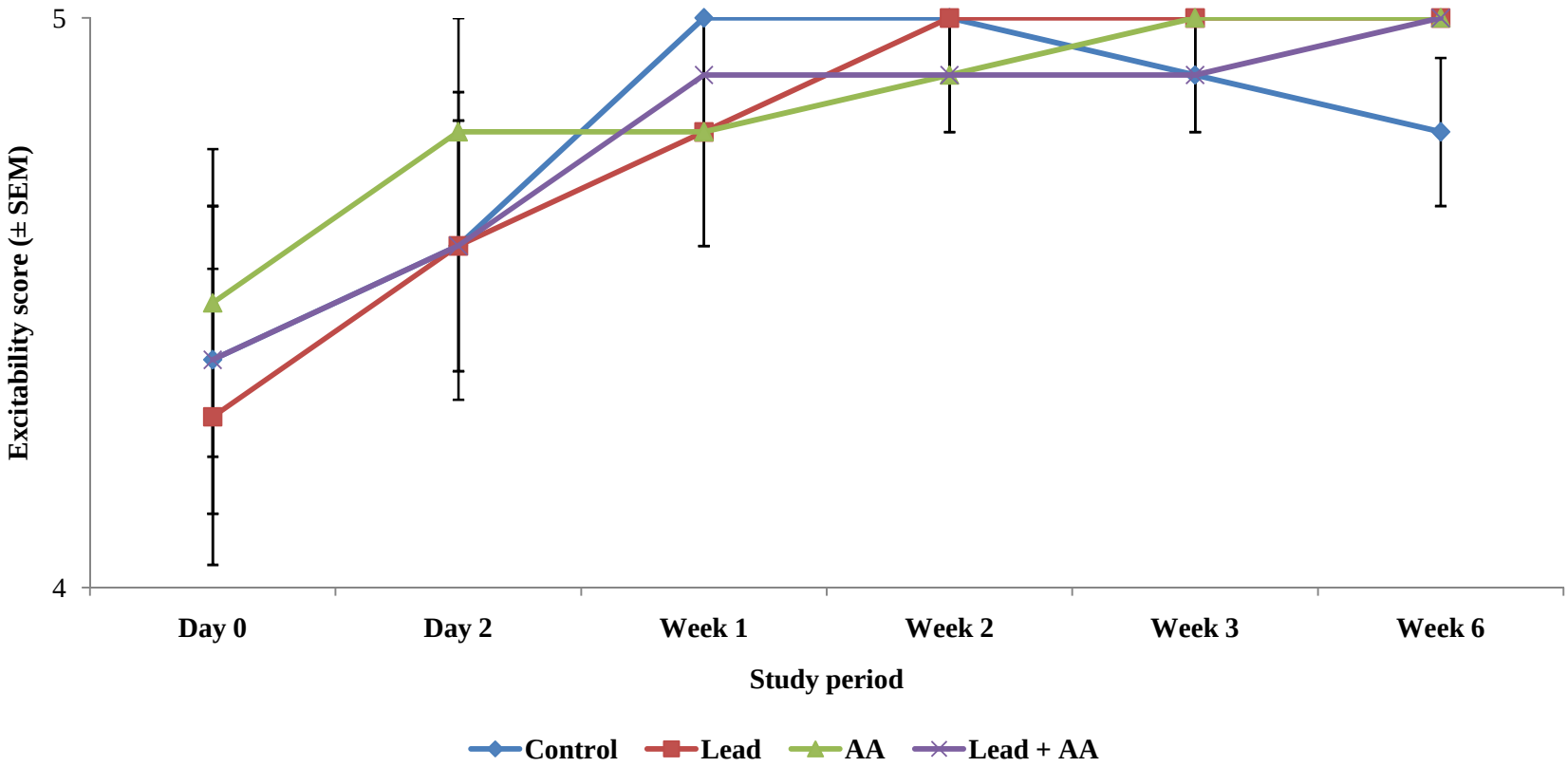


Figure 4.14: Effect of sub-chronic exposure to low-dose level of lead on excitability scores of Wistar rats treated with ascorbic acid. n = 10, AA = Ascorbic acid

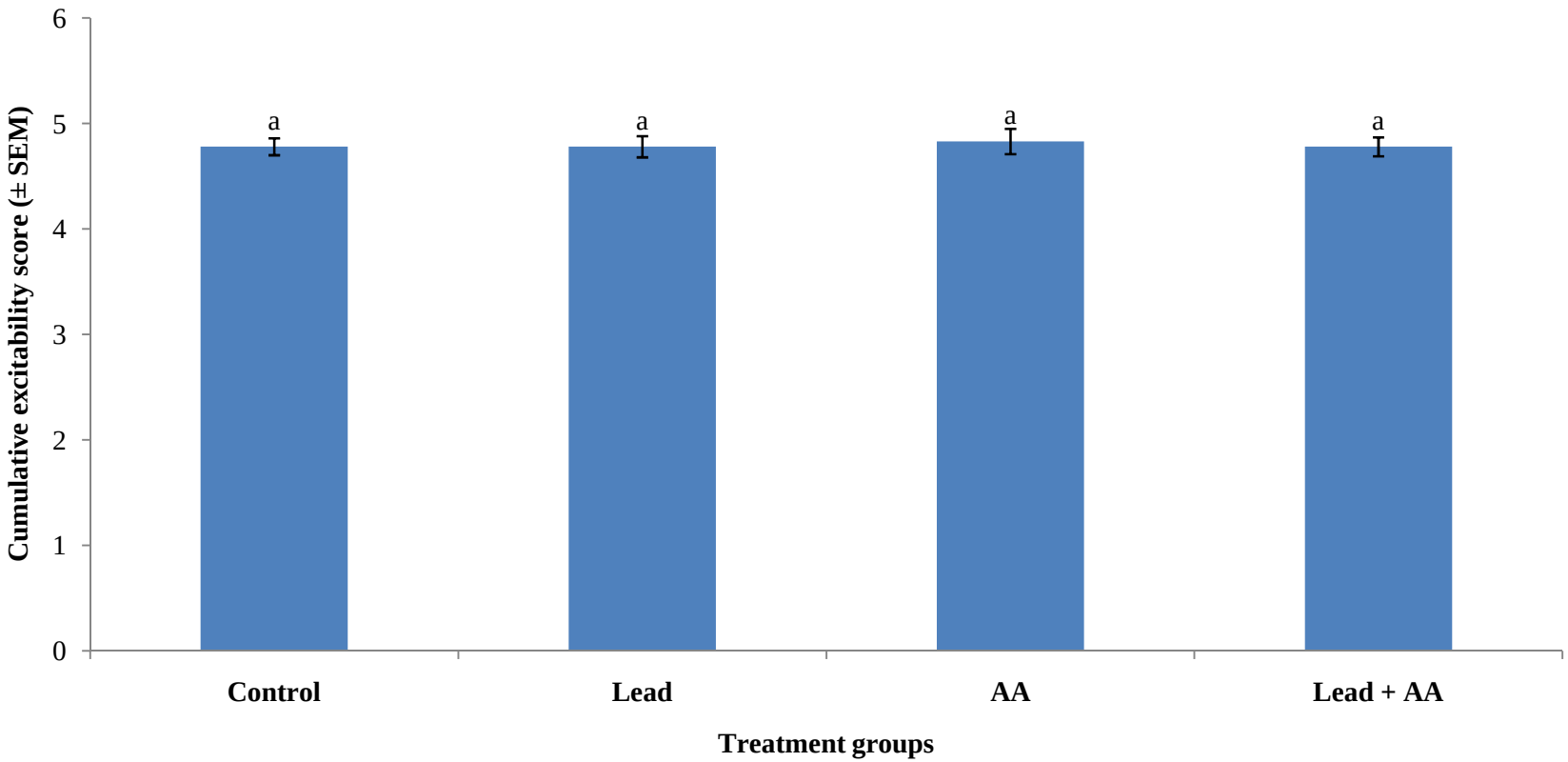


Figure 4.15: Changes in the cumulative mean excitability scores of control (n=10) and experimental (n=10) Wistar rats exposed to low-dose level of lead and treated with ascorbic acid. n = 10, AA = Ascorbic acid. [a] = Values are not significantly (P > 0.05) different.

h. Learning

A significant difference ($P < 0.05$) was observed in learning between the groups. The number of foot-shocks applied to the rats in the lead-treated group (2.80 ± 0.33) was higher compared to that applied to those in the control group (1.20 ± 0.13) and the lead + ascorbic acid-treated group (1.71 ± 0.29) (Figure 4.16). Although the number of foot-shocks applied to the females (2.00 ± 0.0; group III) was higher than that in the males (1.50 ± 0.5; group III), the difference in the values was not significant ($P > 0.05$) (Figure 4.17).

i. Short-term memory

There was no significant difference ($P > 0.05$) in the short-term memory between the groups and sexes. However, a slight decrease was observed in the time spent on the platform in the lead - treated rats, and the value obtained (71.70 ± 14.33 s) was lower than those in the control (104.00 ± 8.58 s), ascorbic acid-treated (107.36 ± 8.27 s) and the lead + ascorbic acid-treated (94.71 ± 15.32 s) groups (Figure 4.18). The males (120.00 ± 0.00 s) spent more ($P < 0.05$) time on the platform than the females in the control group (88.00 ± 14.25 s) (Figure 4. 19).

4.2.4 Effect of sub-chronic administration of lead, ascorbic acid and lead + ascorbic acid on haematological parameters

a. Packed cell volume

There was a significant ($P < 0.05$) decrease in the packed cell volume of rats in the lead-treated group (44.03 ± 1.08 %) compared with the values obtained in rats in the control (47.54 ± 1.02 %), ascorbic acid (49.10 ± 1.09 %) and lead + ascorbic acid (47.70 ± 1.35 %) groups (Figure 4.20).

b. Haemoglobin concentration

There was a significant difference ($P < 0.05$) between the haemoglobin values obtained in lead-treated (13.13 ± 0.35 g/dL), control (14.35 ± 0.21 g/dL) and ascorbic acid-treated (14.55 ± 0.26 g/dL) groups. However, the haemoglobin values obtained in the lead-treated (13.13 ± 0.35 g/dL) and lead + ascorbic acid-treated (13.66 ± 0.25 g/dL) groups were not significantly ($P > 0.05$) different (Figure 4.25).

c. Total erythrocyte count

A significant ($P < 0.05$) decrease was observed in the total erythrocyte value of the lead-treated rats ($7.37 \pm 0.17 \times 10^{12}$/L) compared with values obtained in control ($8.03 \pm 0.18 \times 10^{12}$/L) and the lead + ascorbic acid-treated rats ($8.32 \pm 0.30 \times 10^{12}$/L). The total erythrocyte values obtained in ascorbic acid-treated ($8.41 \pm 0.23 \times 10^{12}$/L) and lead + ascorbic acid - treated ($8.32 \pm 0.30 \times 10^{12}$/L) rats did not differ ($P > 0.05$) (Figure 4.21).

d. Absolute and differential leucocyte counts

The total leucocyte value obtained in lead-treated rats ($6.44 \pm 0.50 \times 10^{6}$ g/dL) when compared with the control group ($5.21 \pm 0.35 \times 10^{6}$ g/dL) was higher ($P < 0.05$) than the corresponding values recorded in control ($5.21 \pm 0.35 \times 10^{6}$ g/dL), ascorbic acid-treated ($4.98 \pm 0.34 \times 10^{6}$ g/dL) and lead + ascorbic acid-treated ($5.23 \pm 0.23 \times 10^{6}$ g/dL) groups (Figure 4.22).

The number of neutrophils in lead-treated group (22.40 ± 1.65 %) was higher ($P < 0.05$) than the value obtained in rats administered with lead + ascorbic acid (16.89 ± 2.04 %). However, a significant ($P < 0.05$) decrease was observed in the number of neutrophils recorded in the control (17.20 ± 1.71 %) and ascorbic acid-treated (19.50 ± 1.51 %) groups when compared to the value recorded in the lead-treated group (22.40 ± 1.65 %). Similarly, the number of lymphocytes in the lead-treated group (73.50 ± 1.47 %) was significantly ($P <$

0.05) lower than that of the control (79.40 ± 2.11 %) and the lead + ascorbic acid-treated (79.89 ± 2.00 %) groups. A slight increase was observed in the ascorbic acid-treated group (75.80 ± 1.96 %) as compared with that of the lead-treated group (73.50 ± 1.47 %). The number of monocytes obtained in the ascorbic acid-treated group (1.70 ± 0.47 %) was significantly ($P < 0.05$) higher in the lead-treated group (0.60 ± 0.27 %) than those in the control (0.30 ± 0.21 %) and the lead + ascorbic acid-treated (0.22 ± 0.22 %) groups. There was no significant ($P > 0.05$) difference in the number of circulating eosinophils and basophils in all the treatment groups (Figure 4.23).

The value of the N/L ratio in the control (0.22 ± 0.03) and the lead + ascorbic acid (0.22 ± 0.03) groups was significantly ($P < 0.05$) higher than those in the lead-treated (0.31 ± 0.03) group. The N/L ratio in the rats treated with ascorbic acid only (0.26 ± 0.02) was slightly ($P > 0.05$) higher than those in the control (0.22 ± 0.03) and lead + ascorbic acid (0.22 ± 0.03) groups (Figure 4.24).

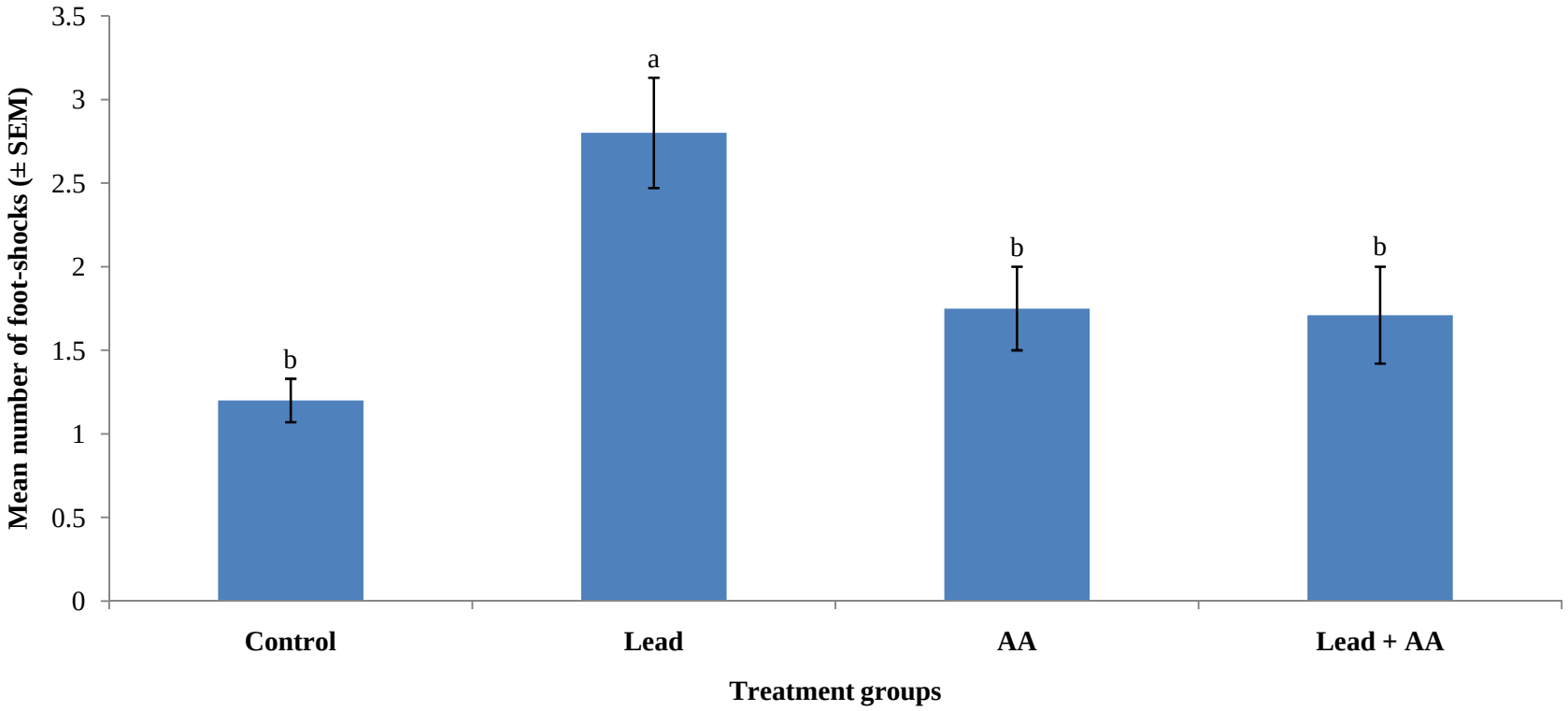


Figure 4.16: Effects of ascorbic acid on learning acquisition in Wistar rats exposed to low-dose level of lead. [a, b] = Values with different superscript letters are significantly (P < 0.05) different. n=10, AA = Ascorbic acid.

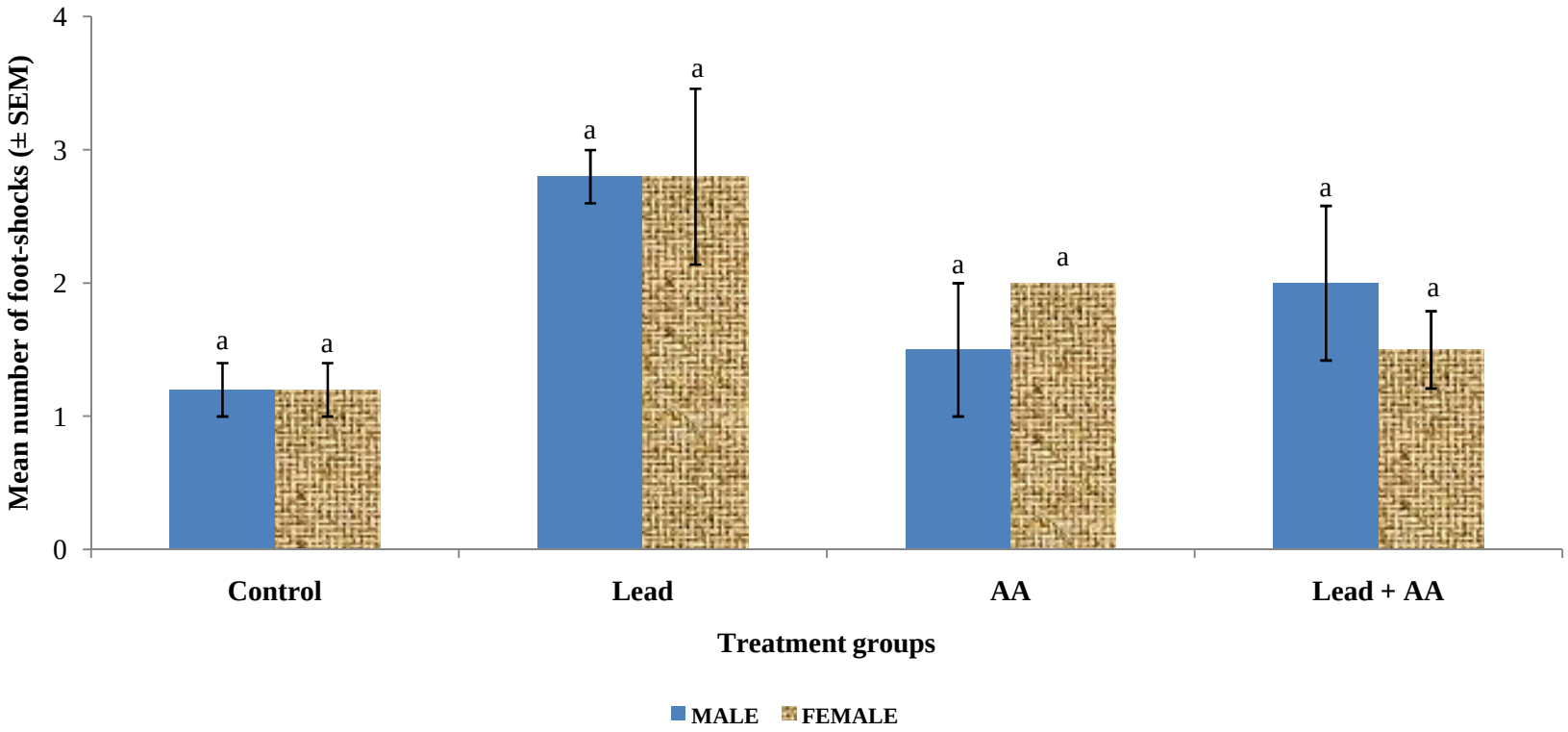


Figure 4.17: Sex variation in responses of Wistar rats exposed to low-dose level of lead and administered with ascorbic acid. AA = Ascorbic acid. [a] = Means with the same letter are not significantly (P > 0.05) different.

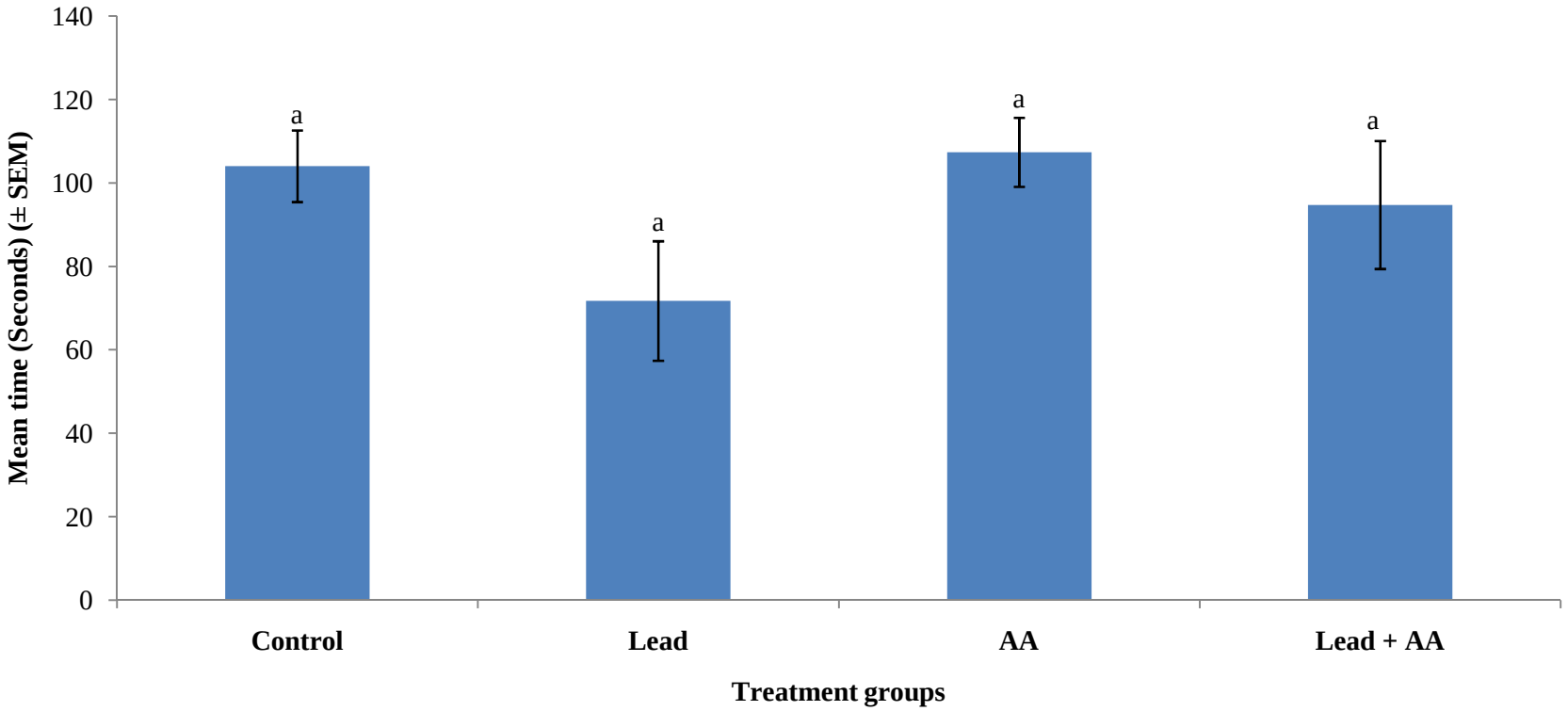


Figure 4.18: Effect of ascorbic acid on short-term memory of rats subjected to sub-chronic low-dose level of lead. n = 10, AA = ascorbic acid. [a] = Means with the same letter are not significantly (P > 0.05) different.

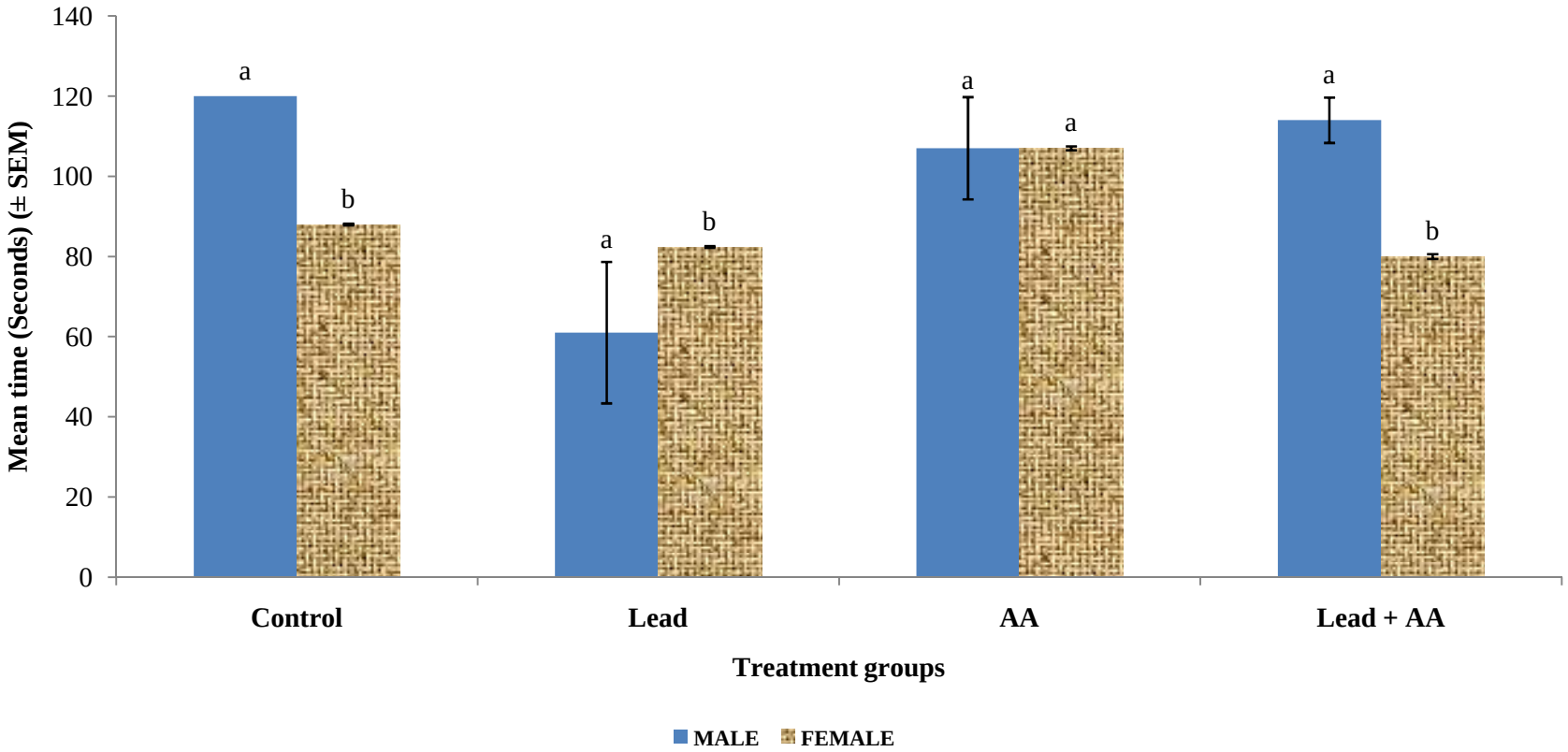


Figure 4.19: Sex variation in short-term memory response of Wistar rats exposed to low-level lead and treated with ascorbic acid. [a, b] = Values with different superscript letters are significantly (P < 0.05) different. AA = ascorbic acid.

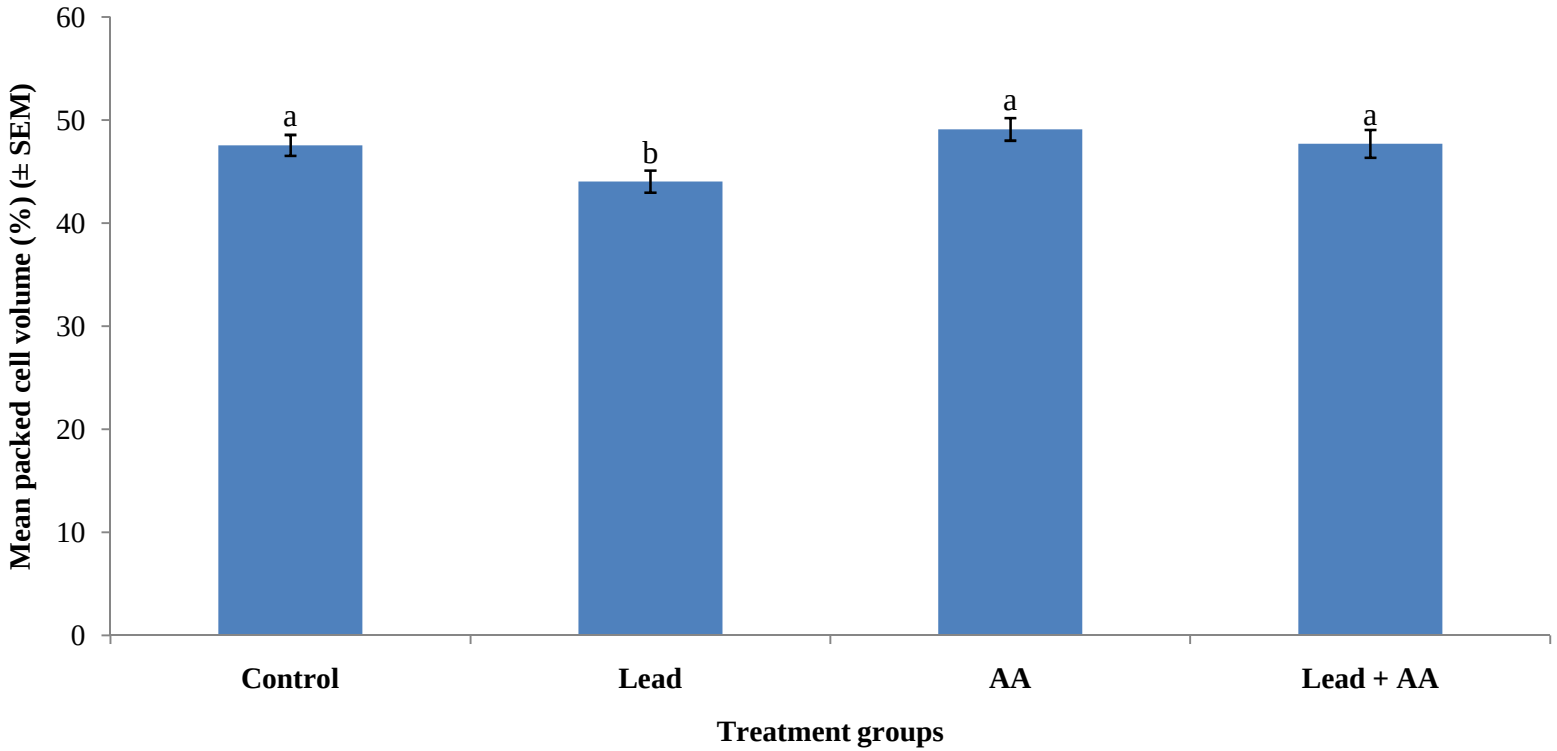


Figure 4.20: Mean changes in packed cell volume of Wistar rats exposed repeatedly to low-dose of lead and treated with ascorbic acid. n = 10, AA = Ascorbic acid. [a, b] = values with different superscript letters are significantly (P < 0.05) different.

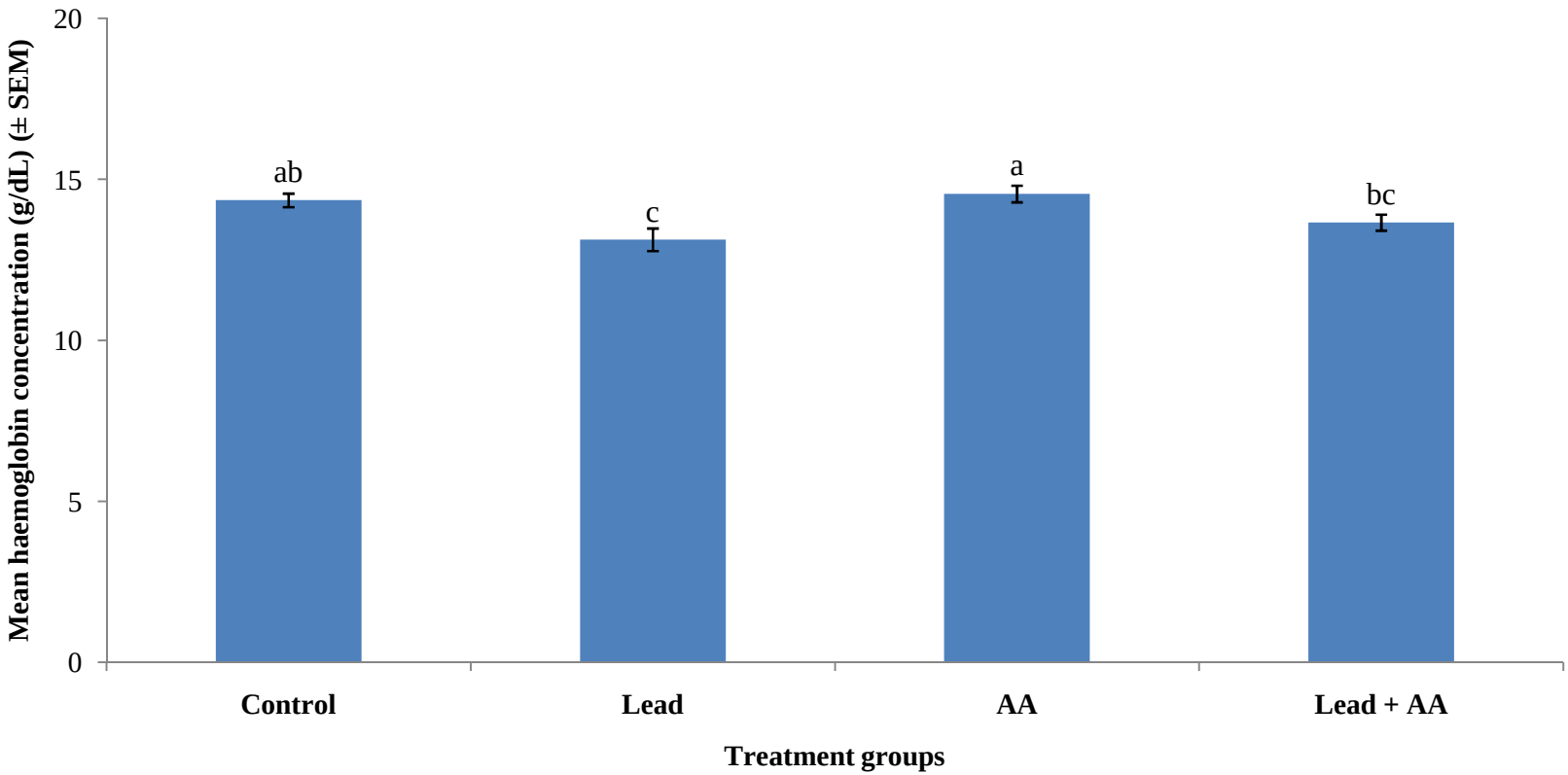


Figure 4.21: Changes in haemoglobin concentration of Wistar rats exposed repeatedly to low-dose lead and treated with ascorbic acid. n = 10, AA = Ascorbic acid. [a, b, c] = Values with different superscript letters are significantly (P < 0.05) different.

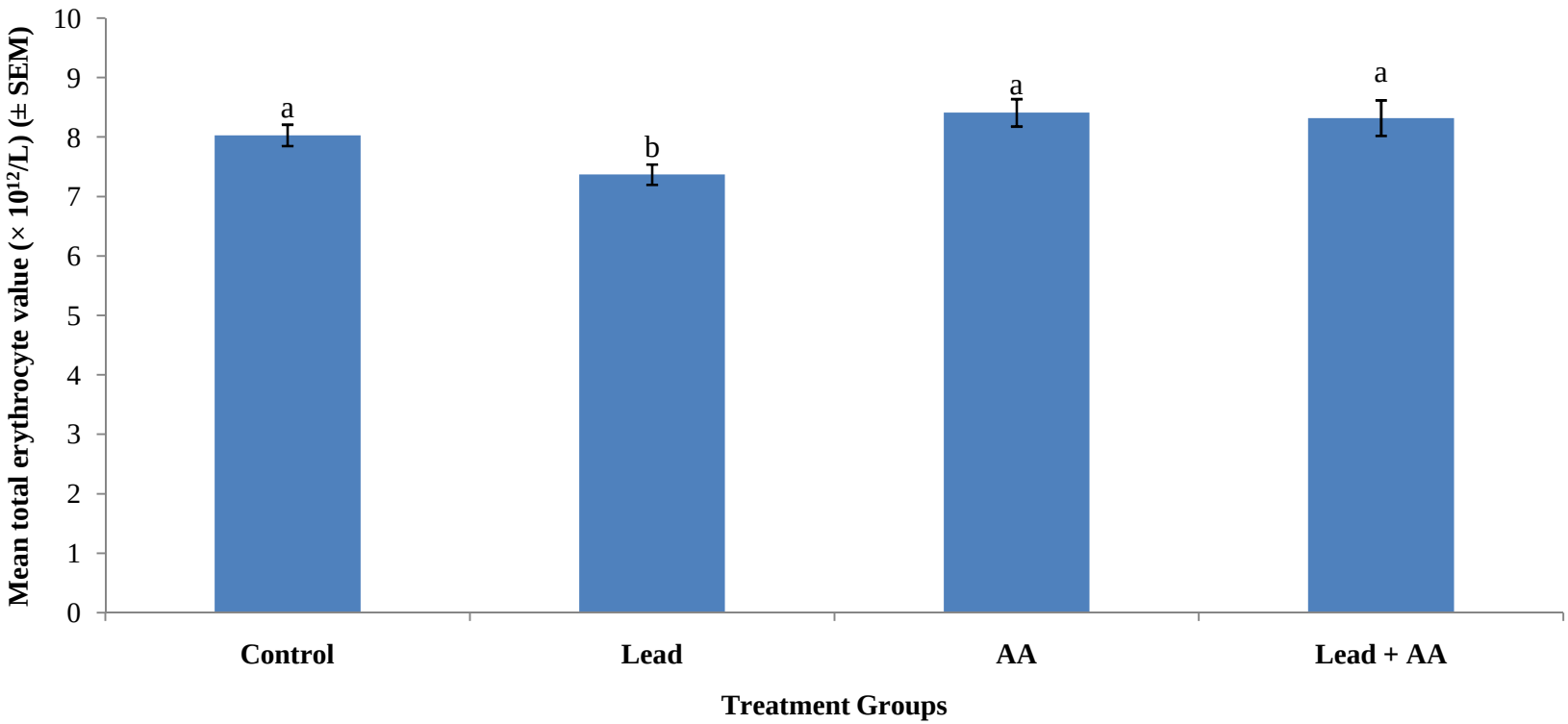


Figure 4.22: Changes in the total erythrocyte count of Wistar rats following repeated exposure to low-dose lead and treatment with ascorbic acid. [a, b] = values with different superscript letters are significantly (P < 0.05) different. n = 10, AA = Ascorbic.

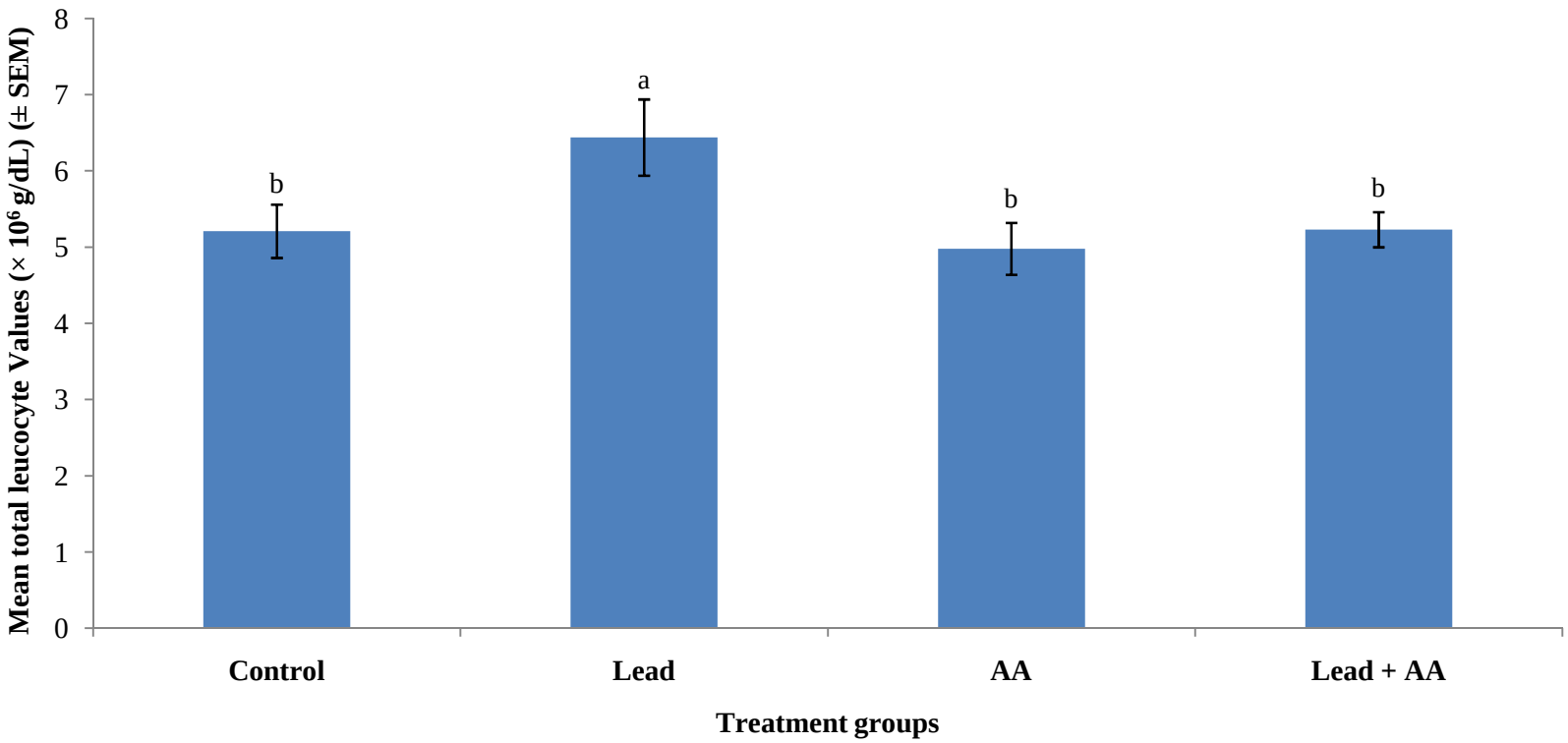


Figure 4.23: Changes in the total leucocyte value of Wistar rats following repeated exposure to low-dose lead and treatment with ascorbic acid. n = 10, AA = Ascorbic acid. [a, b] = Values with different superscript letters are significantly (P < 0.05) different.

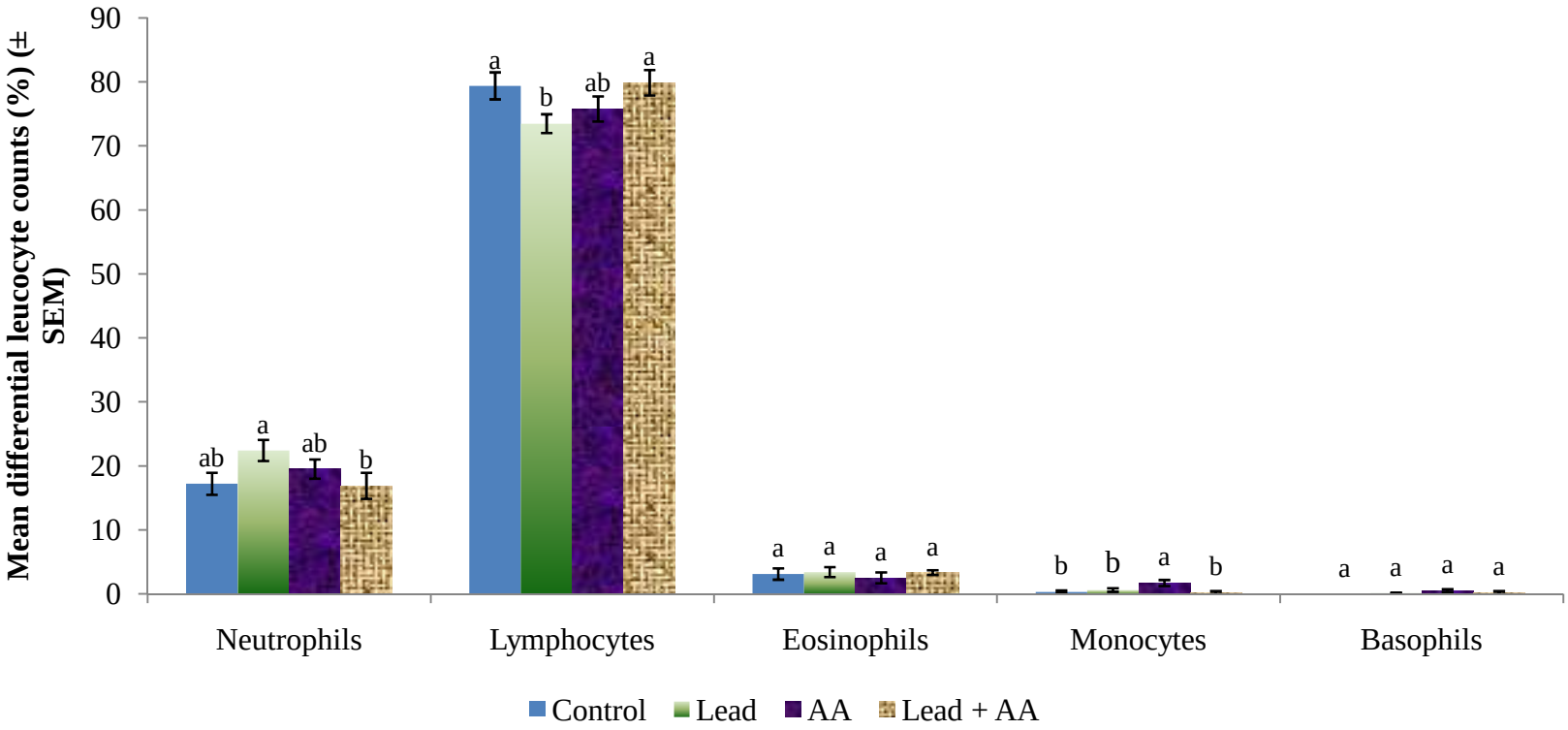


Figure 4.24: Changes in differential leucocyte counts of Wistar rats following repeated exposure to low-dose lead and treatment with ascorbic acid. n = 10, AA = Ascorbic acid. [a, b] = Values with different superscript alphabets are significantly (P < 0.05) different.

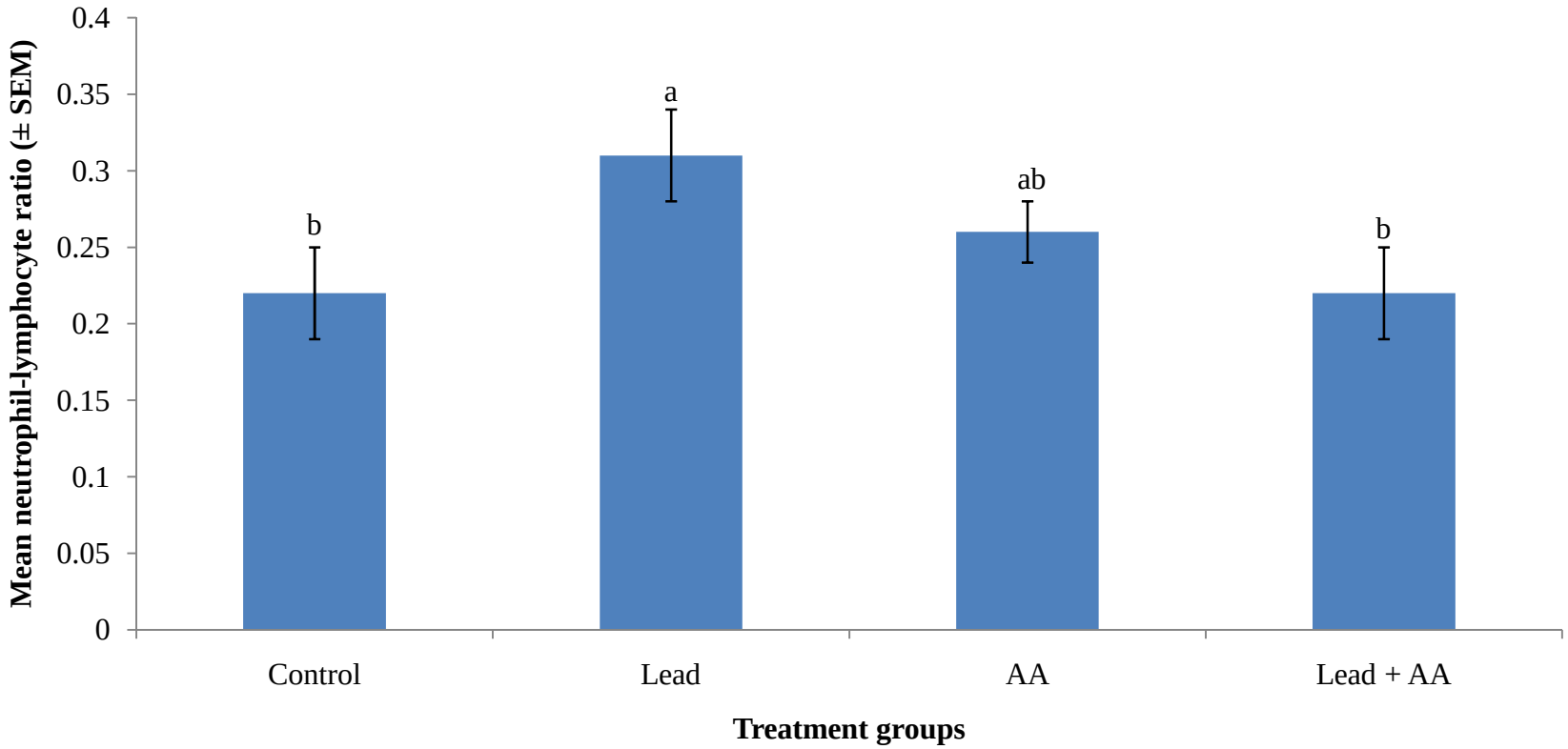


Figure 4.25: Neutrophil/lymphocyte ratio of Wistar rats exposed repeatedly to low-dose lead and treated with ascorbic acid. n = 10, AA = Ascorbic acid. [a, b] = Values with different superscript letters are significantly (P < 0.05) different.

e. *In vitro* erythrocyte osmotic fragility

There was no significant difference ($P > 0.05$) in percent haemolysis of fragility of erythrocytes in all the groups and at the various concentrations of sodium chloride. However at 0.5 % NaCl concentration, there was a slight insignificant increase in the percent haemolysis of erythrocytes in the lead-treated group (9.65 ± 2.14 %) and the lead + ascorbic acid-treated group (11.16 ± 1.21 %), compared with the control group (8.43 ± 1.18 %) and the ascorbic acid-treated group (7.11 ± 0.83 %) (Figure 4.26).

4.2.5 Effect of sub-chronic administration of lead, ascorbic acid, and lead + ascorbic acid on serum biochemical tests

a. AST, ALT, ALP and LDH

A significant difference ($P < 0.05$) was observed in the serum activity of aspartate aminotransferase between the groups, but no significant ($P > 0.05$) variation was recorded in the activity of alanine aminotransferase between the groups. There was a decrease in the activity of aspartate aminotransferase in the lead-treated group (161.50 ± 9.65 IU/L) as compared with the control group (225.70 ± 16.59 IU/L) and the lead + ascorbic acid group (196.56 ± 7.31) (Figure 4.27). However, a slight decrease in the activity of alanine aminotransferase was observed in rats treated with only lead (91.10 ± 6.68 IU/L), compared with those in the control group (96.00 ± 6.59 IU/L). A further slight decrease was observed in the lead + ascorbic acid-treated group (85.00 ± 8.39 IU/L). However, the values were not significant ($P > 0.05$) (Figure 4.26). There was no significant ($P > 0.05$) difference in the activity of serum alkaline phosphatase between the groups. Rats treated with lead only (193.90 ± 6.73 IU/L) had a slight increase in alkaline phosphatase compared with the control group (189.70 ± 17.62 IU/L) (Figure 4.27).

A significant difference ($P < 0.05$) was observed in the activity of lactate dehydrogenase between the groups, but not between the sexes. There was a decrease ($P < 0.05$) in the activity of lactate dehydrogenase in the lead-treated group (1077.70 ± 57.66 IU/L) compared with the control group (1835.70 ± 204.68 IU/L). Similarly, a slight increase in the activity of this enzyme was observed in the lead + ascorbic acid-treated group (1485.56 ± 56.41 IU/L), compared with the lead-treated group (1077.70 ± 57.66 IU/L) (Figure 4.28).

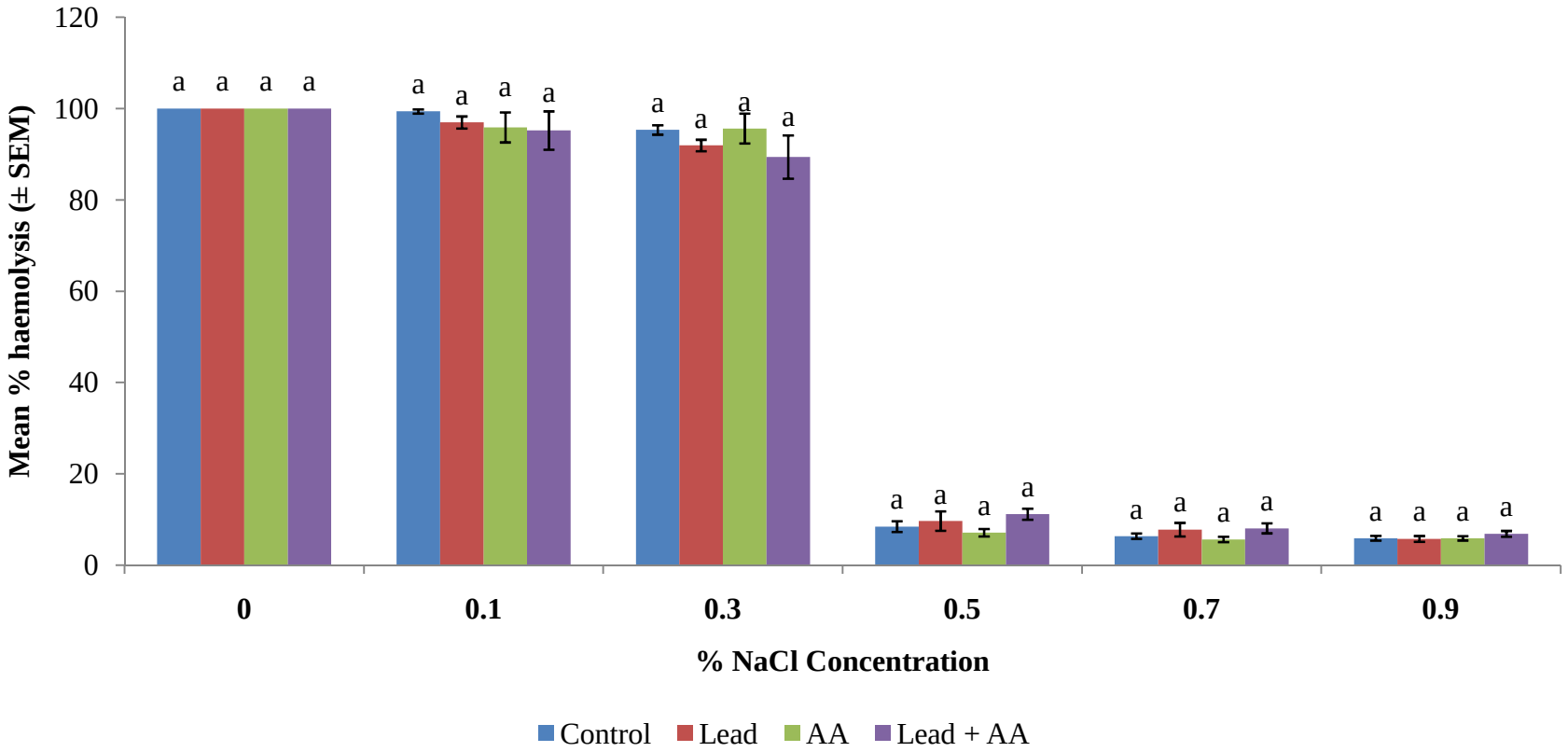


Figure 4.26: Changes in the osmotic fragility of erythrocytes in Wistar rats repeatedly exposed to low-dose lead and treated with ascorbic acid. [a] = Values with the same superscript letters are not significantly (P > 0.05) different. n = 10, AA = Ascorbic acid.

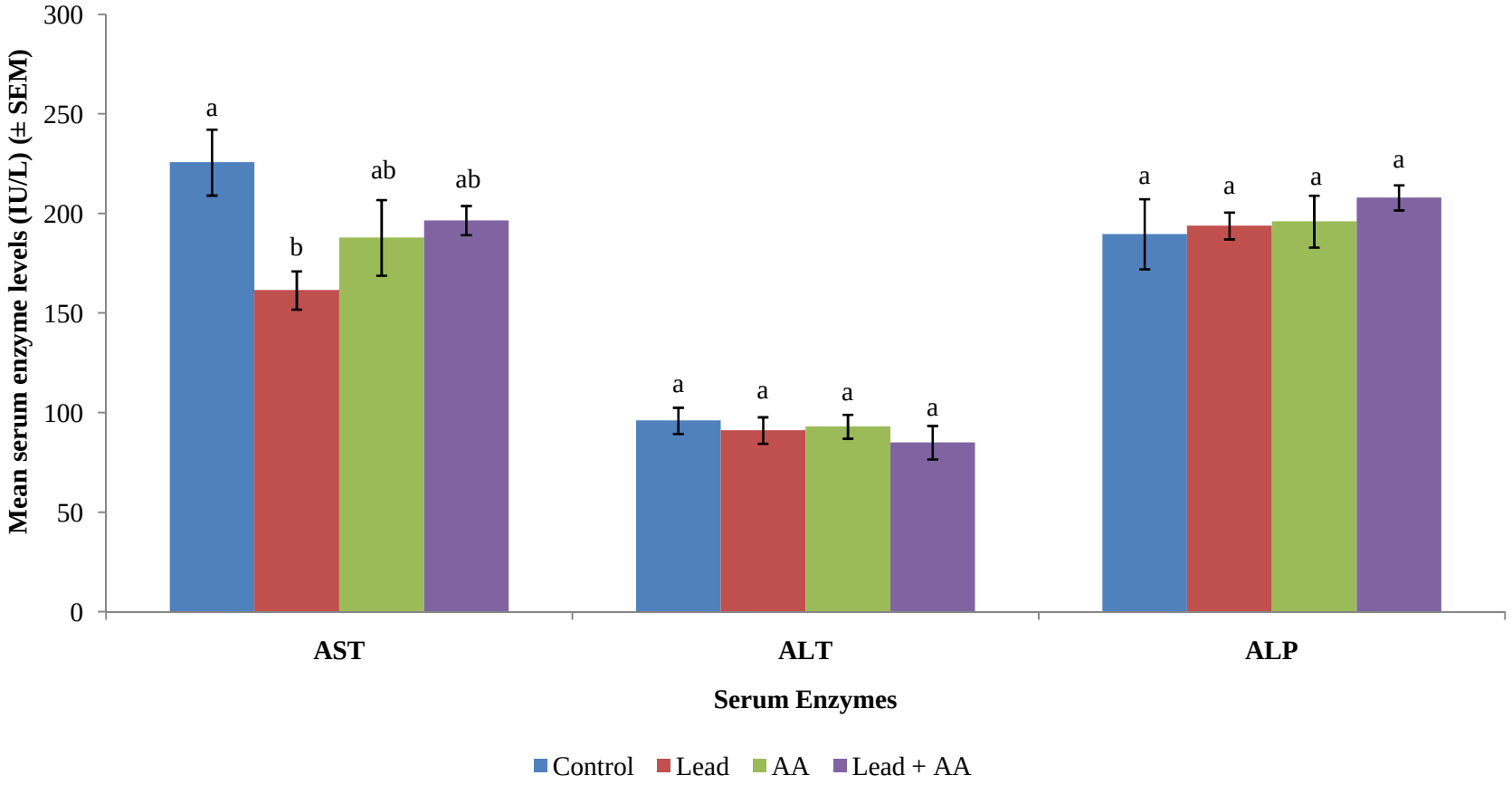


Figure 4.27: Changes in serum enzyme levels of rats administered low-dose level of lead acetate and treated with ascorbic acid. [a, b] = values with different superscript alphabets are significantly (P < 0.05) different. n = 10, AA = ascorbic acid.

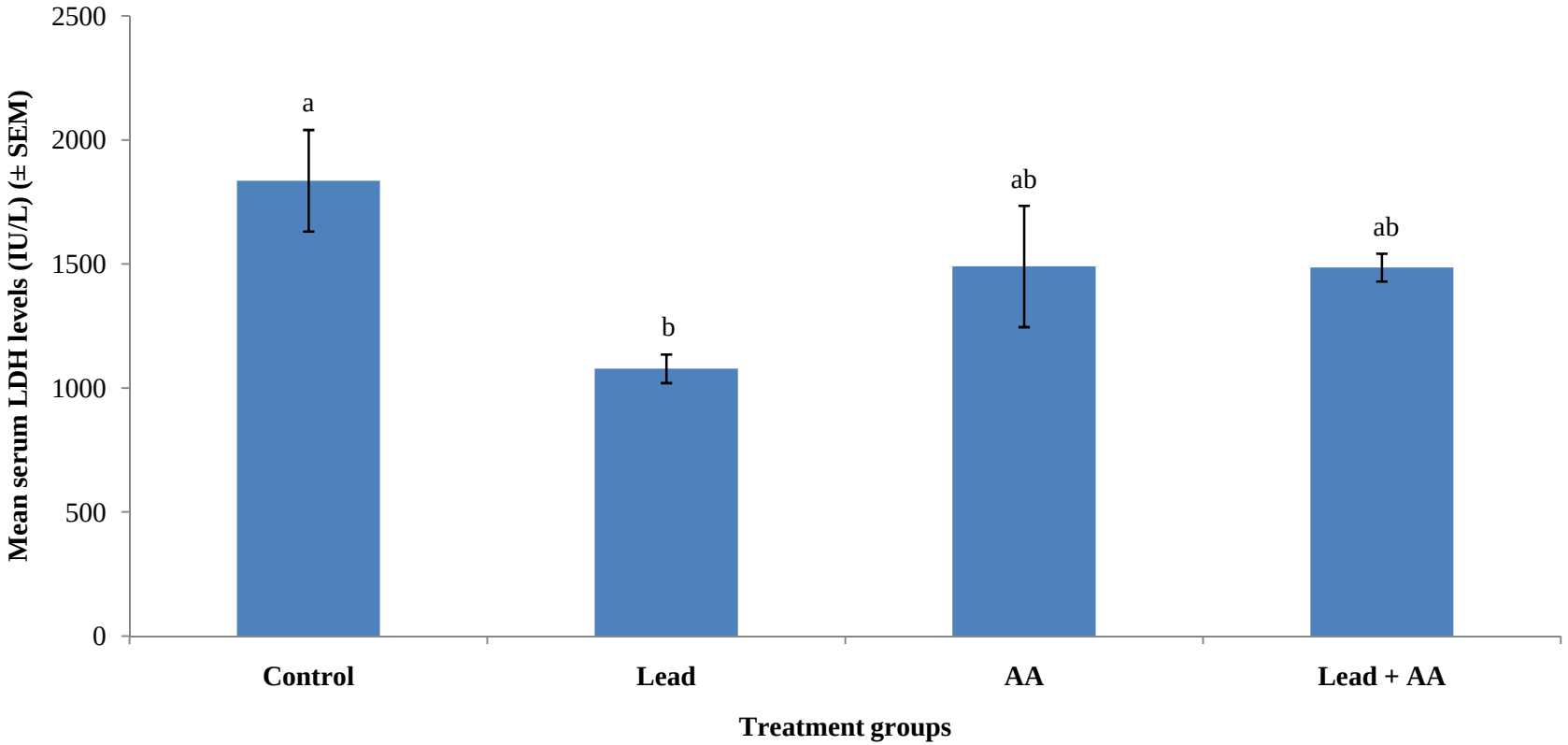


Figure 4.28: Mean serum levels of Lactate dehydrogenase in rats repeatedly exposed to lead acetate and pretreated with ascorbic acid. [a, b] = values with different superscript alphabets are significantly (P < 0.05) different. n = 10, AA = ascorbic acid.

b. Creatine kinase

A significant difference ($P < 0.05$) was observed between the groups and sexes. There was a decrease in rats treated with lead only (1408.70 ± 274.18 IU/L) as compared with that of the control group (2263.40 ± 430.73 IU/L). Similarly, a significant increase ($P < 0.05$) was observed in the ascorbic acid-treated rats (2635.10 ± 314.95 IU/L) and in rats administered with lead + ascorbic acid (3333.20 ± 408.71 IU/L) as compared with those treated with lead (1408.70 ± 274.18 IU/L) (Figure 4.29).

c. Serum proteins

There was a significant ($P < 0.05$) difference in the serum total protein concentration between the groups. A significant ($P < 0.05$) decrease was observed in the lead-treated group (80.50 ± 1.14 g/L), compared with the lead + ascorbic acid-treated group (84.20 ± 1.79 g/L) (Figure 4.30). There was no significant difference ($P > 0.05$) in the serum albumin concentrations between the groups (Figure 4.30).

The serum globulin level in the lead-treated group (42.11 ± 0.90 g/L) showed a slight increase, compared to those in the control (40.67 ± 1.73 g/L). However, there was a significant ($P < 0.05$) increase in the serum globulin concentration of the animals treated with only ascorbic acid (45.89 ± 1.49 g/L), compared with the rats in the control (40.67 ± 1.73 g/L) and those in the lead-treated group (42.11 ± 0.90 g/L). The rats also administered with lead + ascorbic acid (44.33 ± 1.39 g/L) had a slightly higher serum globulin levels than those treated with only lead (42.11 ± 0.90 g/L) (Figure 4.30).

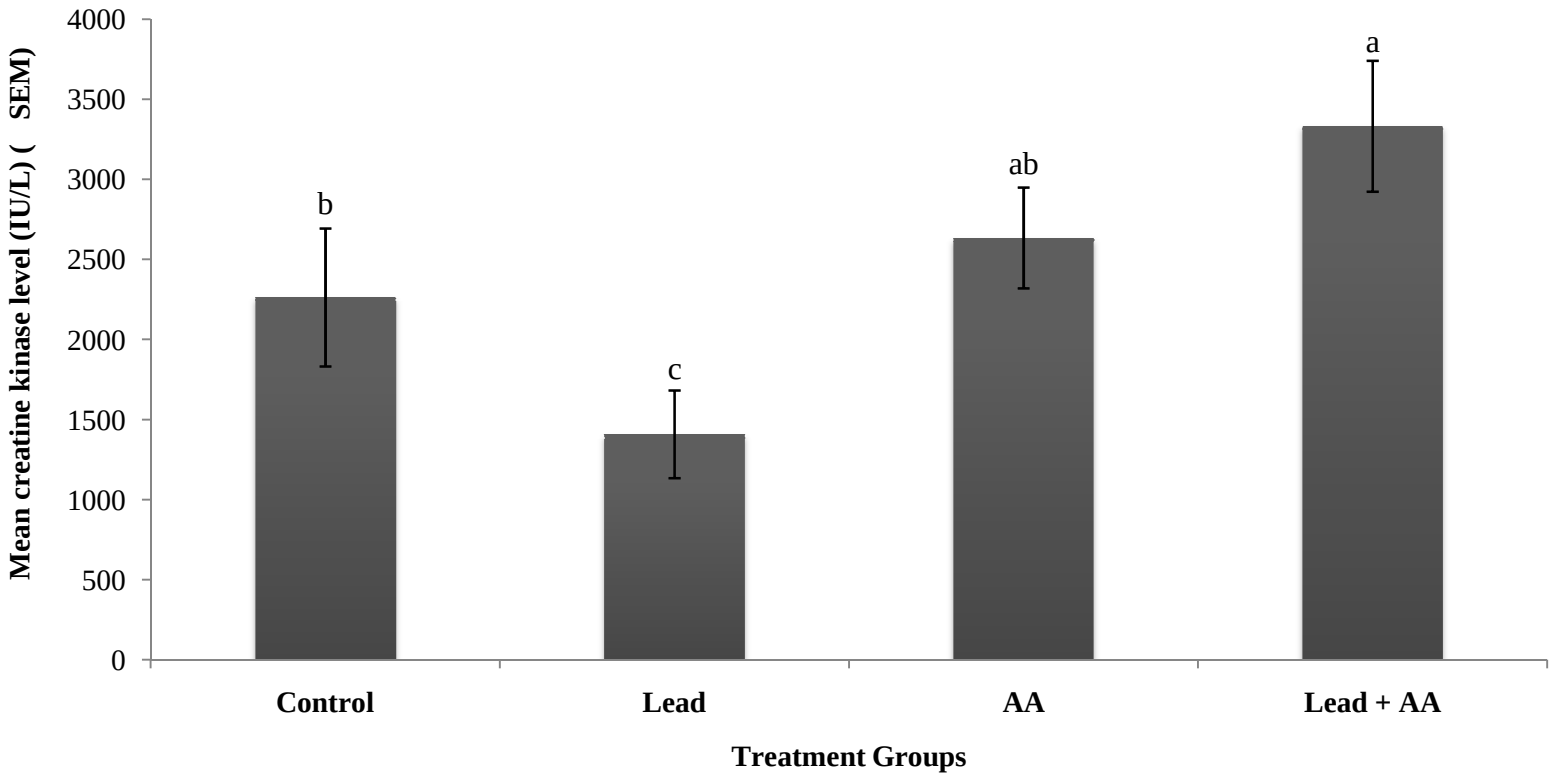


Figure 4.29: Effects of ascorbic acid on serum creatine kinase levels of Wistar rats exposed to low-level of lead for six weeks. n = 10, $^{a, b, c}$ = Values with different superscript letters are significantly (P < 0.05) different. AA = Ascorbic acid

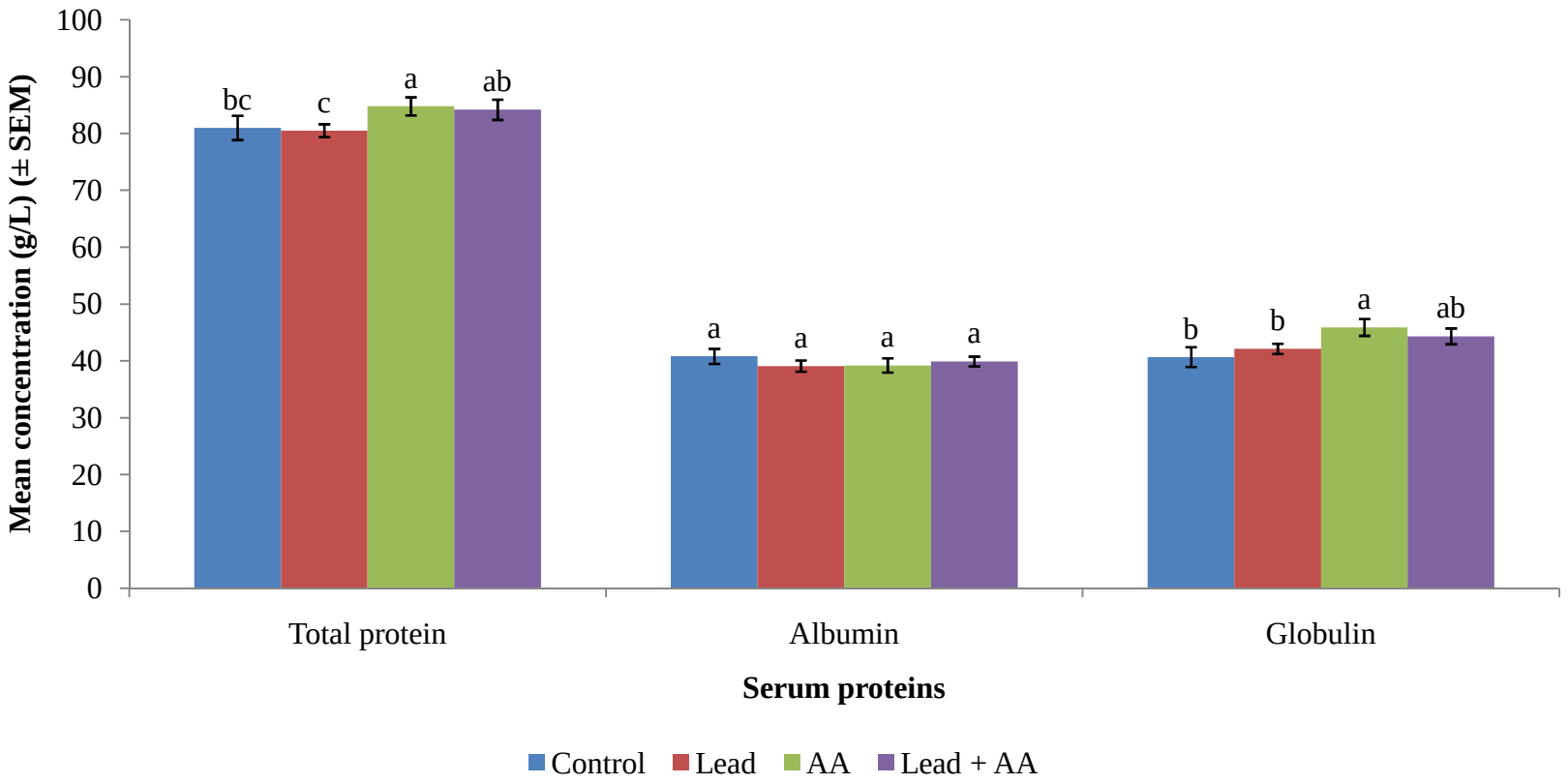


Figure 4.30: Effect of ascorbic acid on serum total protein, albumin and globulin concentration of Wistar rats exposed to sub- chronic repeated low-dose level of lead acetate. [a, b, c] = Values with different superscript letters are significantly (P < 0.05) different. n = 10, AA = Ascorbic acid.

d. Urea concentration

There was no significant difference ($P > 0.05$) in urea concentration between the groups. There was however, an increase in the lead-treated group (7.10 ± 0.41 mmol/L) compared to those of the control group (7.00 ± 0.53 mmol/L) and the lead + ascorbic acid - treated group (6.44 ± 0.56mmol/L) (Table 4.2).

e. Creatinine and uric acid concentration

There was no significant difference ($P > 0.05$) in the concentration of serum creatinine between the groups. Similarly, it was observed that there was no significant difference ($P > 0.05$) in the concentration of uric acid between the groups. A slight decrease observed in the lead-treated group (88.40 ± 6.58 µmol/L) compared with the control (95.40 ± 5.78 µmol/L), the ascorbic acid-treated (98.20 ± 6.72) and the lead + ascorbic acid-treated groups (95.11 ± 7.26 µmol/L) were not significant ($P > 0.05$) (Table 4.2).

Table 4.2: Effects of treatments on creatinine, uric acid and urea concentrations in Wistar rats.

Parameters	Treatment groups			
	Control	**Lead**	**Ascorbic acid**	**Lead + ascorbic acid**
Creatinine (µmmol/L)	81.50 ± 4.94[a]	82.8 ± 6.09[a]	84.20 ± 5.44[a]	82.44 ± 6.15[a]
Uric acid (µmmol/L)	95.40 ± 5.78[a]	88.40 ± 6.58[a]	98.20 ± 6.72[a]	95.11 ± 7.26[a]
Urea (mmol/L)	7.00 ± 0.53[a]	7.10 ± 0.41[a]	6.60 ± 0.52[a]	6.44 ± 0.56[a]

n = 10, [a] = Values with the same superscript alphabets are not significantly ($P > 0.05$) different.

f. Brain malondialdehyde concentration

A significant ($P < 0.05$) decrease was observed in the brain malondialdehyde (MDA) concentration of the ascorbic acid-treated group (6.77 ± 0.76 mmol/mg protein) compared with the control group (9.53 ± 0.73 mmol/mg protein). In addition, the lead + ascorbic acid group (7.66 ± 0.73 mmol/mg protein) also showed a decrease in the brain MDA concentration compared with the lead-treated group (9.63 ± 0.44 mmol/mg protein). A slight but insignificant increase in the brain MDA concentration of the lead-treated rats (9.63 ± 0.44 mmol/mg protein) was recorded compared with the rats in the control group (9.53 ± 0.73 mmol/mg protein) (Figure 4.31)

4.2.6 Effect of sub-chronic administration of lead acetate, ascorbic acid, and lead acetate + ascorbic acid on thyroid function

a. Total serum triiodothyronine concentration

There was no significant ($P > 0.05$) difference in total triiodothyronine concentration between the groups and sexes. However, an insignificant ($P > 0.05$) decrease was obtained in the lead-treated rats (0.91 ± 0.09 ng/ml), compared to those in the control (1.04 ± 0.15 ng/ml) and the lead + ascorbic acid-treated groups (1.27 ± 0.09 ng/ml) (Figure 4.32).

b. Total serum thyroxine concentration

There was no significant ($P > 0.05$) difference between the groups and the sexes. As with total triiodothyronine, a slight decrease was observed in the lead-treated group (3.60 ± 0.22 µg/ml), compared with the control group (3.84 ± 0.32 µg/ml) and the lead + ascorbic acid-treated group (3.68 ± 0.29 µg/ml) (Figure 4.32).

c. Total serum thyrotropin concentration

No significant ($P > 0.05$) difference was observed in the concentration of thyrotropin between the groups and sexes. An insignificant increase was, however, observed in the lead-treated group (0.49 ± 0.03 µIU/ml), compared with the control group (0.45 ± 0.02 µIU/ml). A corresponding increase was observed in the concentration of thyrotropin in the ascorbic acid-treated (0.56 ± 0.04 µIU/ml) and the lead + ascorbic acid-treated groups (0.50 ± 0.04 µIU/ml), compared with the control group (0.45 ± 0.02 µIU/ml) (Figure 4.32).

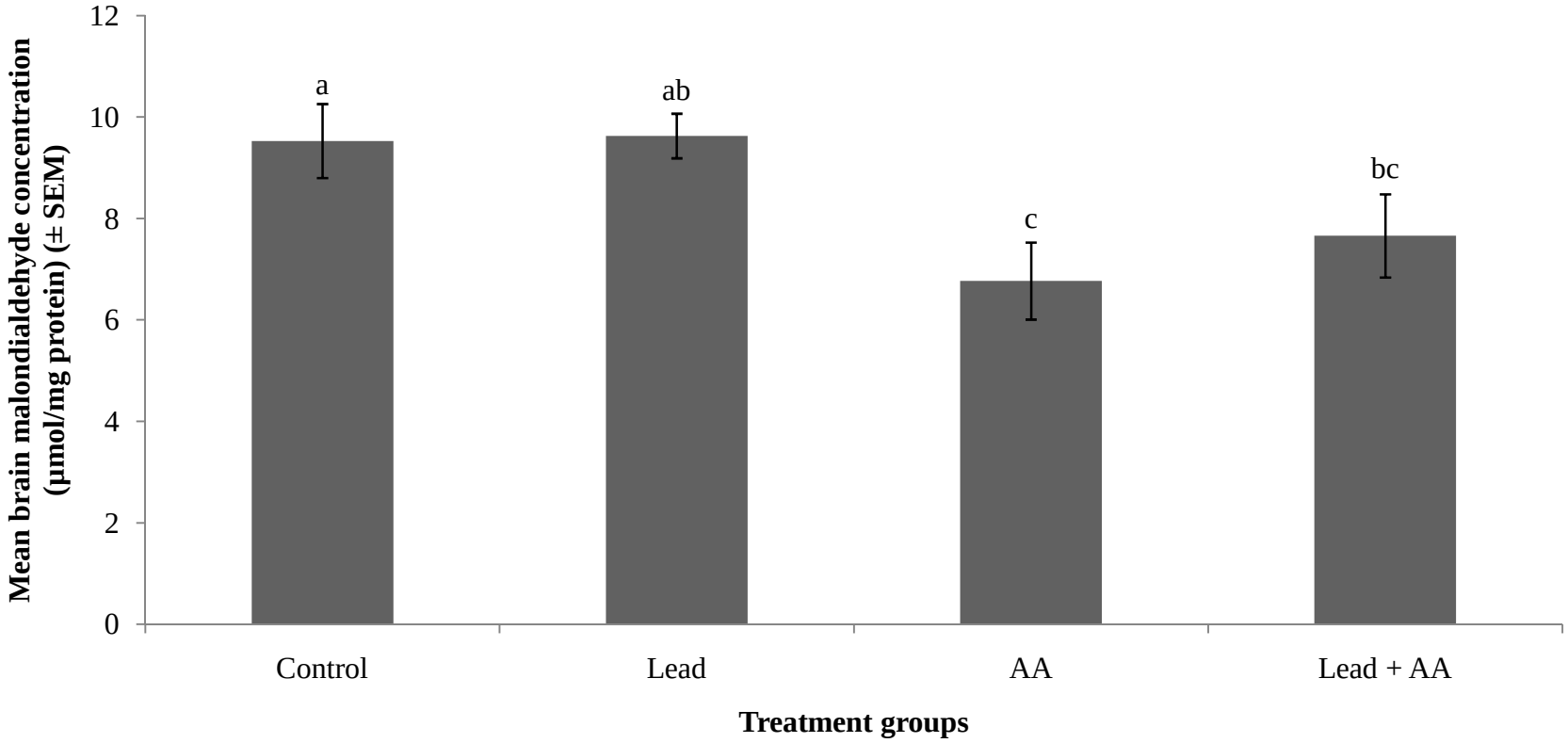


Figure 4.31: Effect of sub-chronic administration of repeated low-dose lead acetate on the brain malondialdehyde concentration in Wistar rats treated with ascorbic acid. n = 5, AA = Ascorbic acid. [a, b, c] = Values in the same tissue with different superscript letters are significantly (P < 0.05) different.

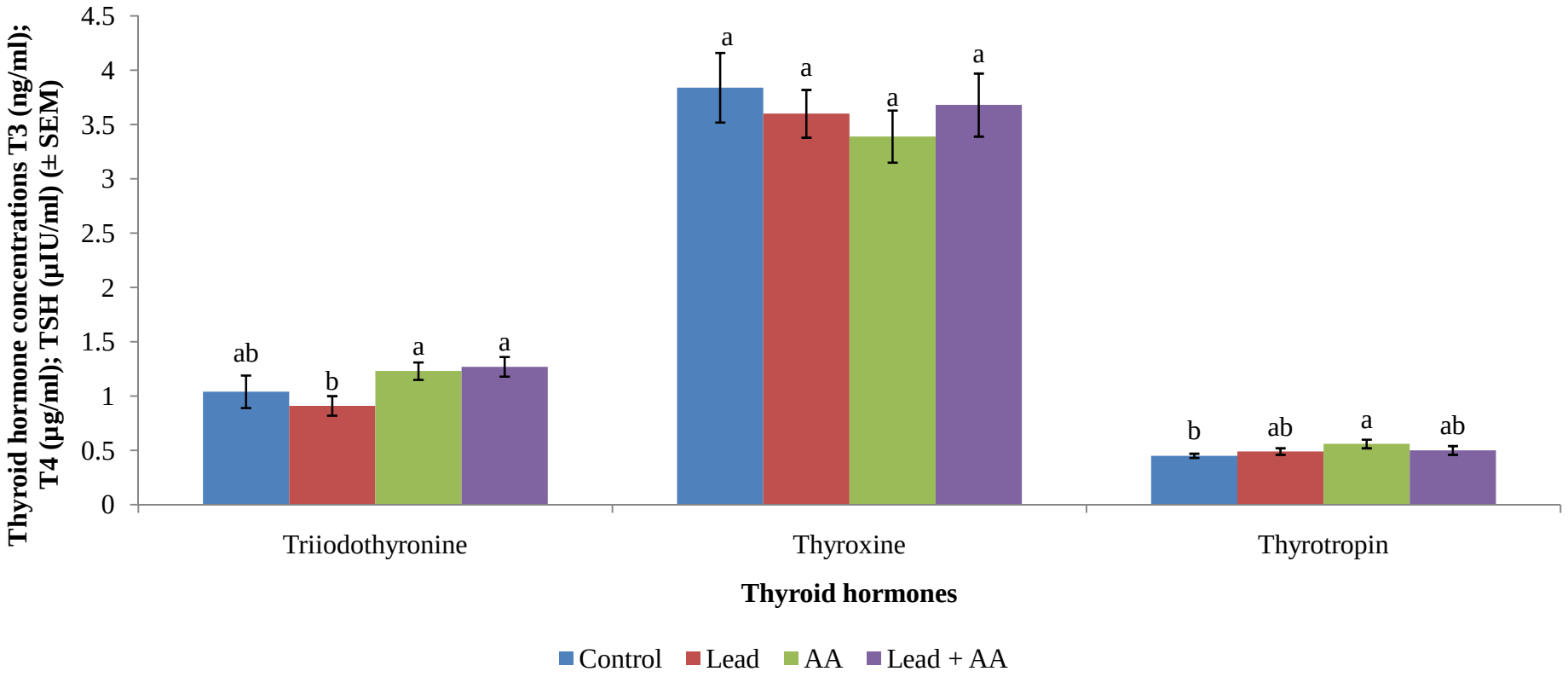


Figure 4.32: Mean changes in the concentration of triiodothyronine, thyroxine and thyrotropin in Wistar rats following sub-chronic exposure to repeated low-dose lead and treatment with ascorbic acid. [a, b] = Values with different superscript letters are significantly (P < 0.05) different. n = 10, AA = Ascorbic acid.

4.2.7 Effect of sub-chronic administration of lead acetate, ascorbic acid, and lead acetate + ascorbic acid on tissue pathology

a. Gross lesions associated with treatments

There were no observable gross pathologic lesions in rats in the control group. Rats exposed to lead only showed congestion of brain vessels, congestion and enlargement of liver, kidneys and thyroid gland. The gross pathologic lesions shown by rats in the lead + ascorbic acid group were slight enlargement and congestion of the liver, kidneys and thyroid gland with congestion of the brain vessels.

b. Histopathological changes

i. Brain

Rats in the control group had no observable microscopic lesions. Histologic lesions in the brain of rats exposed to lead only were microvascular prominence, with congestion and loss of neurones due to karyohexes and pyknosis. There was complete loss of nuclear material characteristic of necrosis, leading to vacuolation. Rats pretreated with ascorbic acid and exposed to lead showed prominence of microvessels without congestion and hypertrophy of endothelial lining of the meninges.

ii. Liver

No observable microscopic lesion occurred in rats in the control group. Rats in the lead-treated group showed diffuse haemorrhagic foci within hepatic sinosoids, and oedema in sinosoids. There was no observable microscopic lesion in the liver of rats in the lead + ascorbic acid group.

iii. Kidneys

There was a loss of normal architecture of the glomeruli leading to almost complete disappearance of the glomeruli in the groups treated with lead acetate only. Rats in the lead + ascorbic acid group showed diffuse haemorrhages on the cortical area and glomeruli.

iv. Thyroid gland

There was no observable microscopic lesion in rats in the control group. Diffuse haemorrhagic foci were observed in the rats exposed to lead acetate only. Rats in the lead + ascorbic acid group showed hyperplasia of the secretory ducts (follicles).

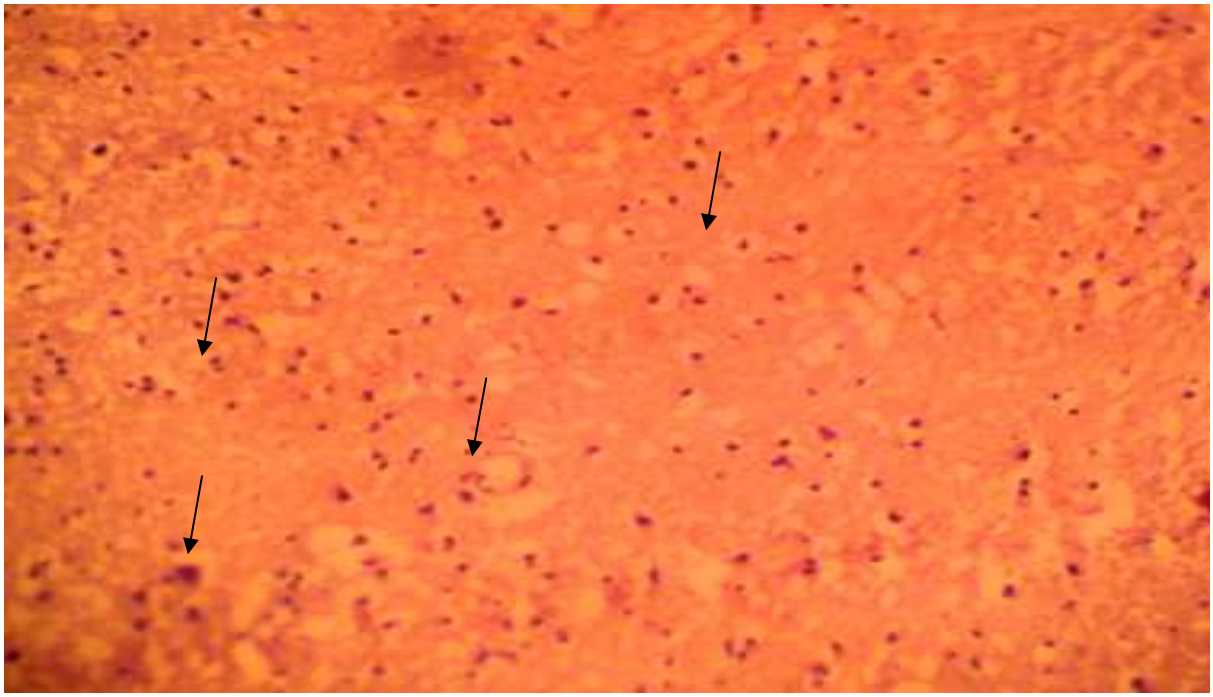


Plate I: Section of brain of rats administered distilled water (arrows show neurones at different stages of differentiation). H & E × 100

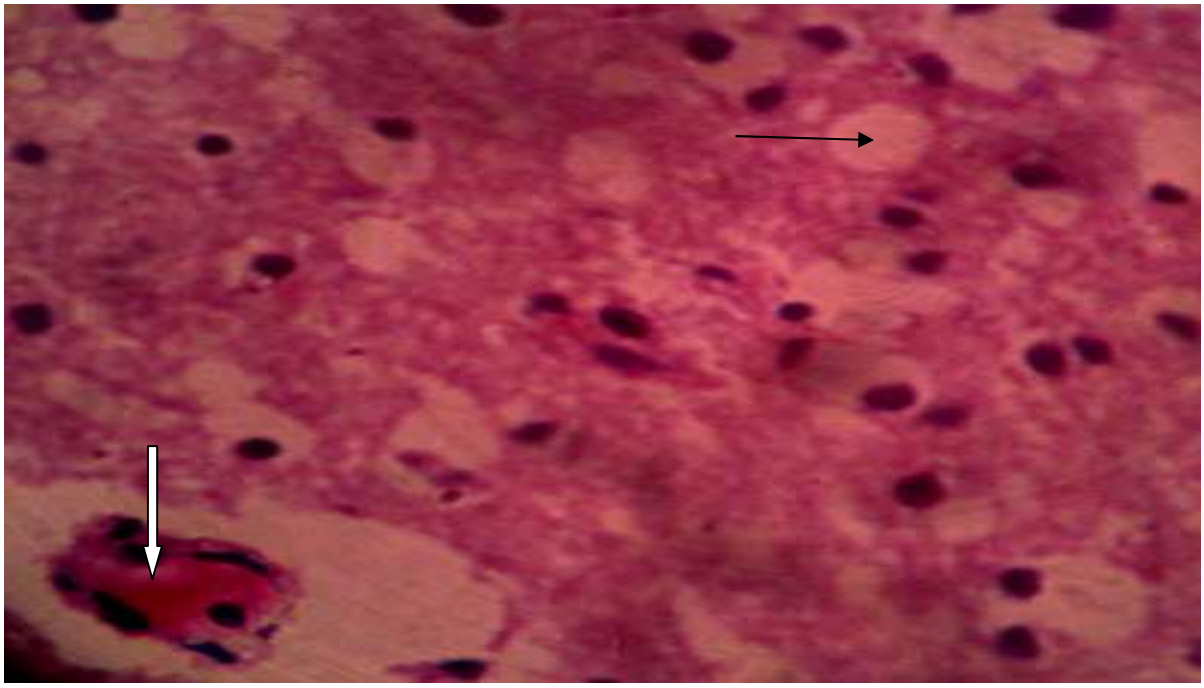


Plate II: Section of brain of rats dosed with lead acetate only showing microvascular prominence associated with congestion (block arrow), loss of neurones due to necrosis leading to vacuolation (line arrow). H & E × 400

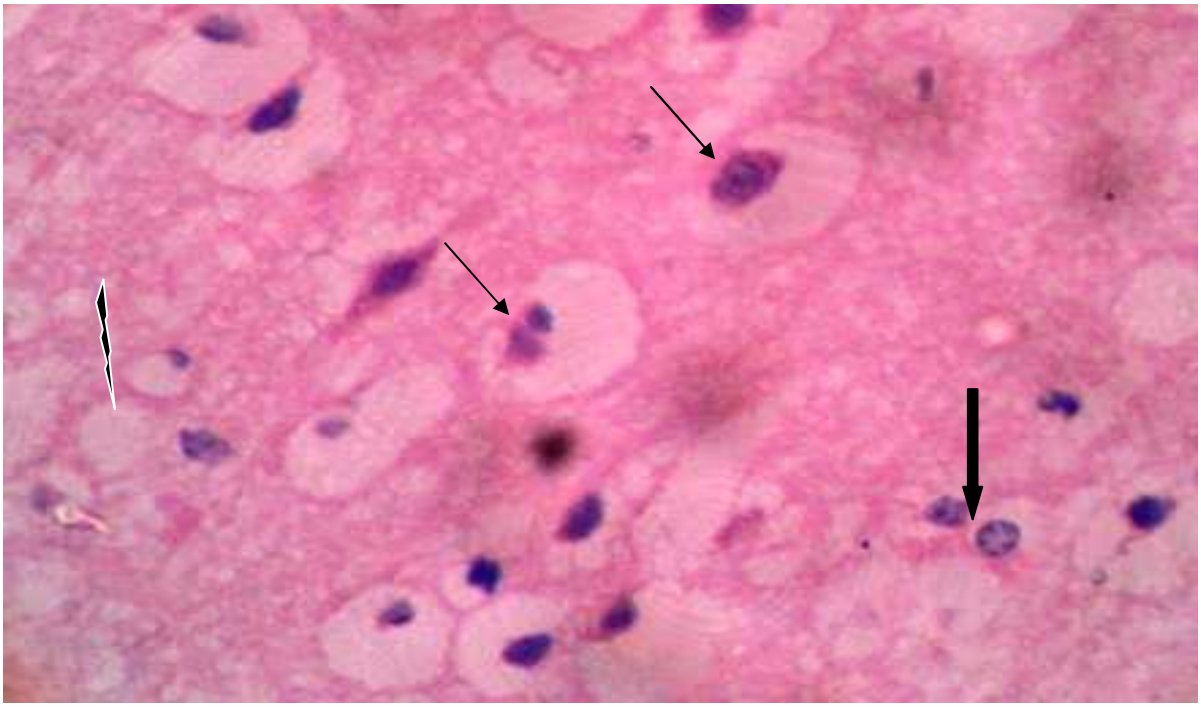


Plate III: Section of brain of rats exposed to lead acetate showing several neurones seen at various stages of cell degeneration, with karyohexes (block arrows), pyknosis (line arrows) and in some cases complete loss of nuclear material characteristic of necrosis (lightning bolts). H & E × 400

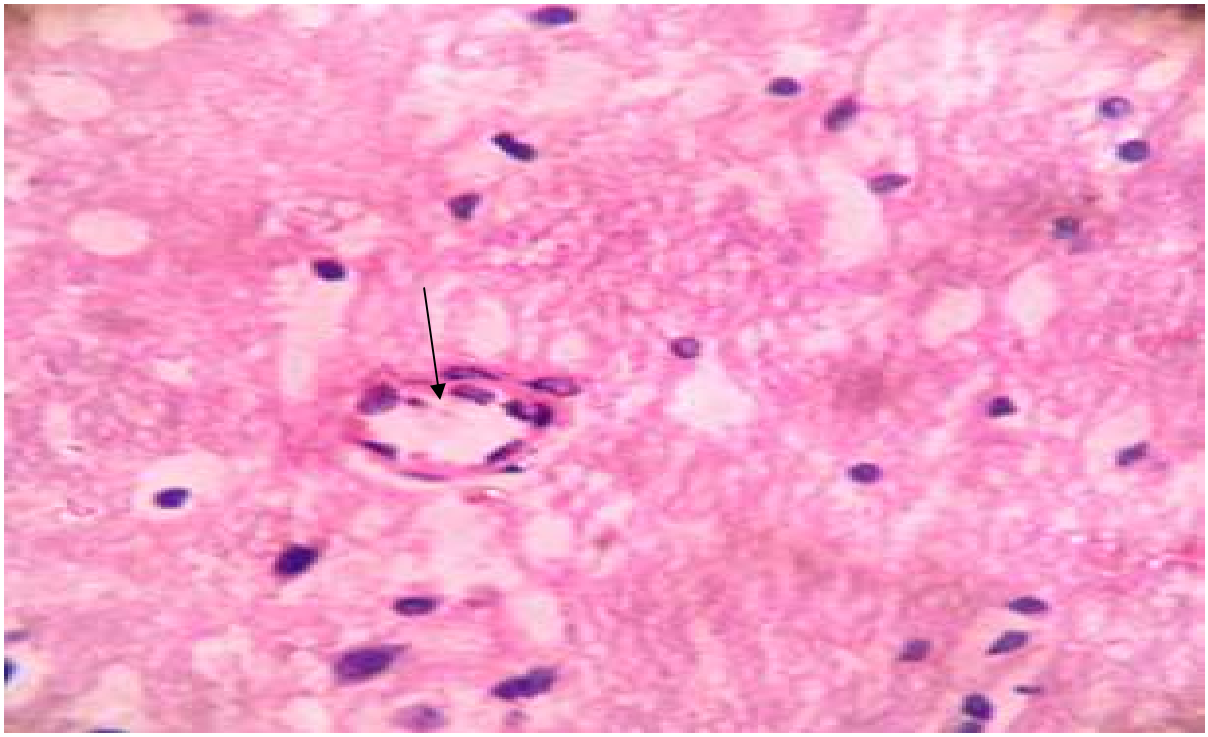


Plate IV: Section of rat brain exposed to pretreated with ascorbic acid and exposed to lead showing prominence of microvessels. H & E × 400

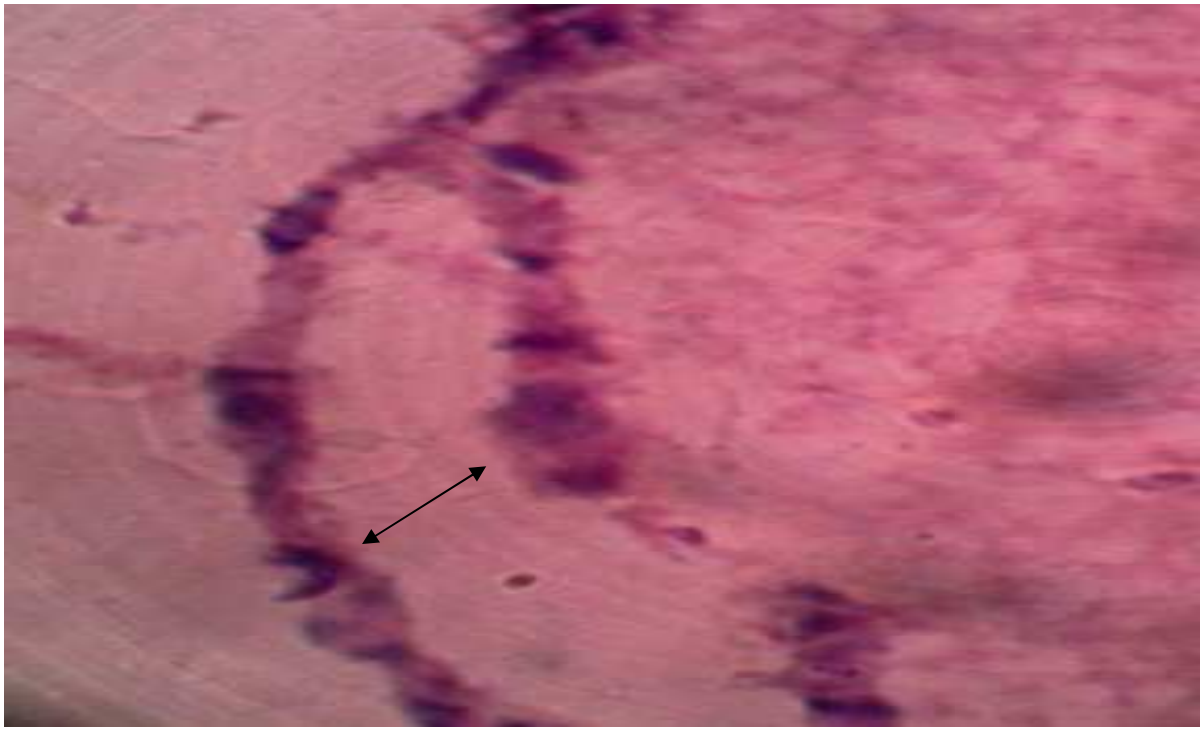


Plate V: Section of rat brain pre-treated with ascorbic acid and exposed to lead acetate showing hypertrophy of endothelial lining of the meninges. H & E × 400

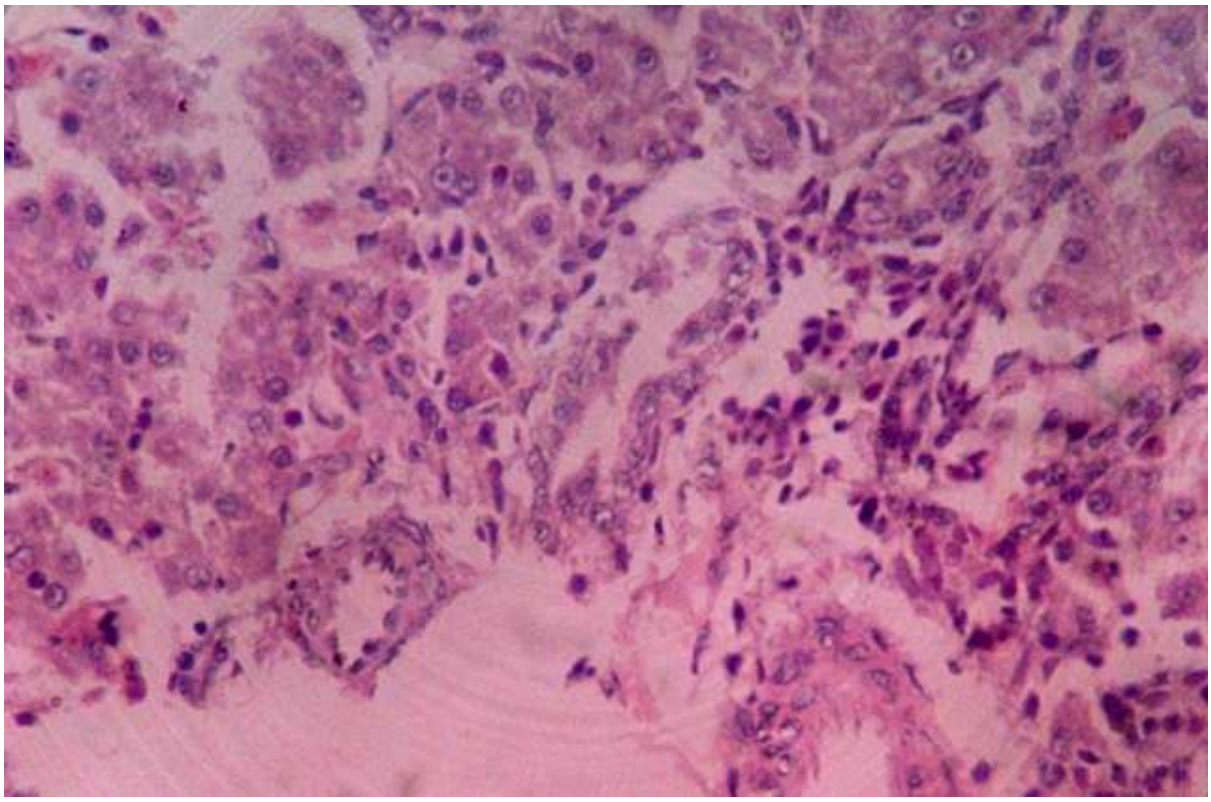


Plate VI: Section of liver of rats administered distilled water showing normal microscopic architecture. H & E × 400

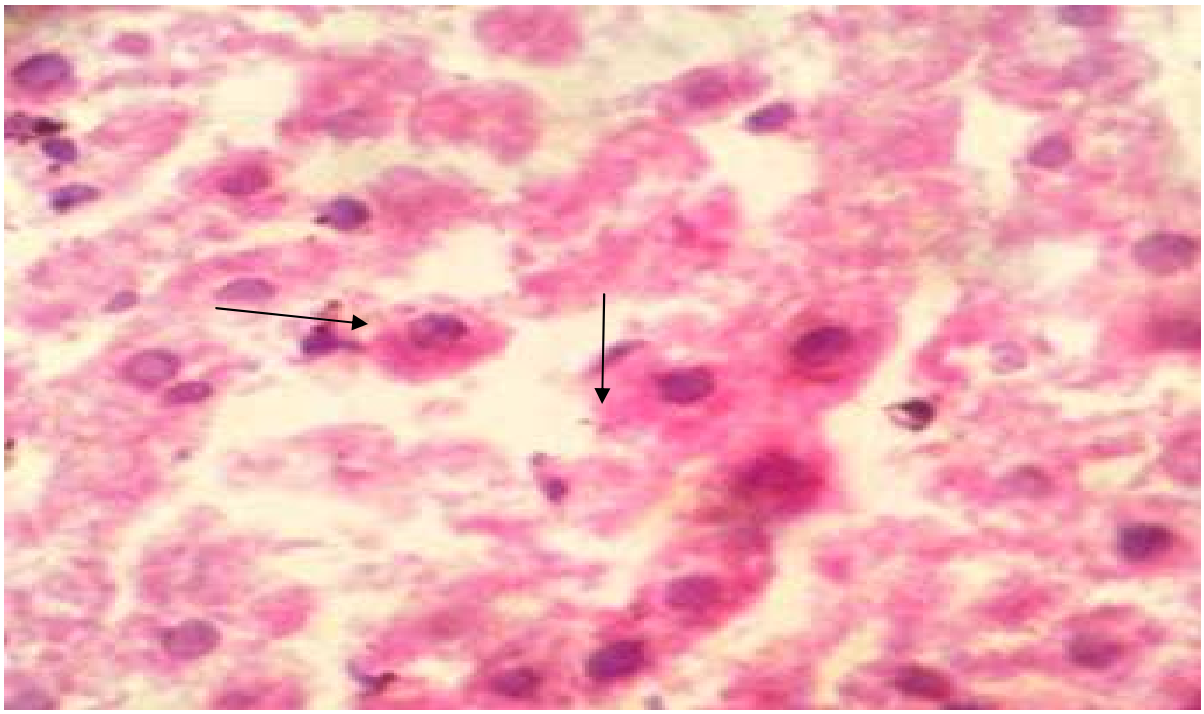


Plate VII: Section of the liver of rats exposed to lead showing diffuse foci of haemorrhages within hepatic sinosoids (line arrows) and total loss of microscopic architecture. H & E × 400

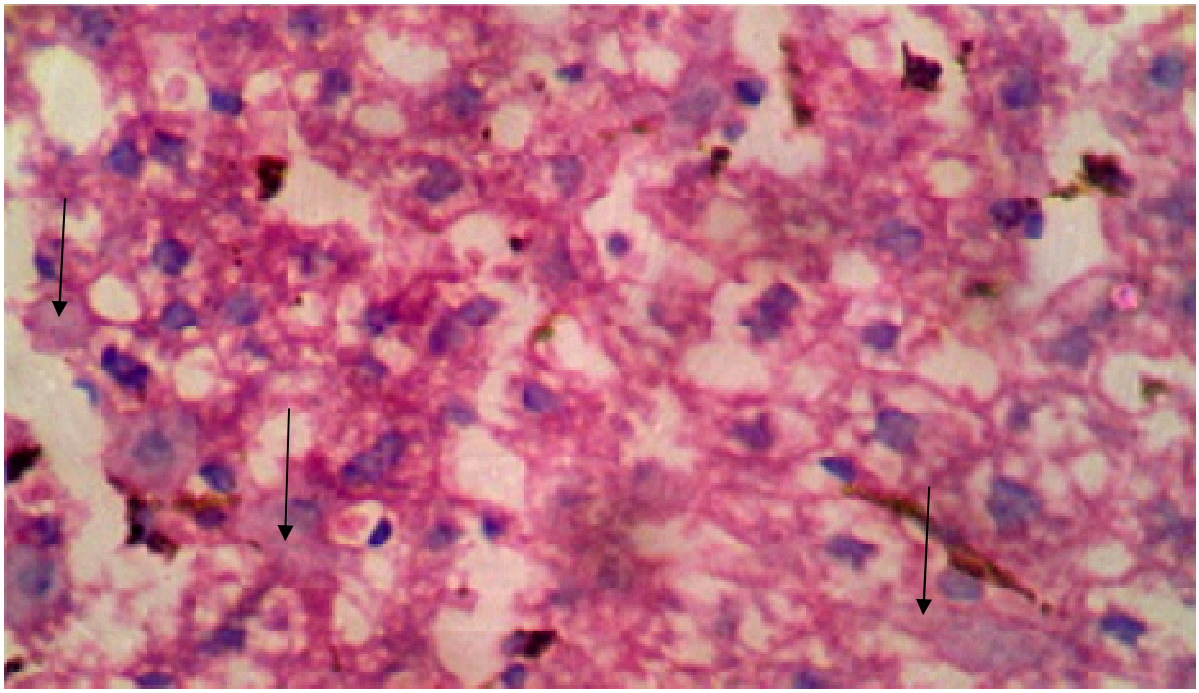


Plate VIII: Section of liver of rats pretreated with ascorbic acid and exposed to lead acetate only, showing oedema in sinosoids (bold arrows). H & E × 400

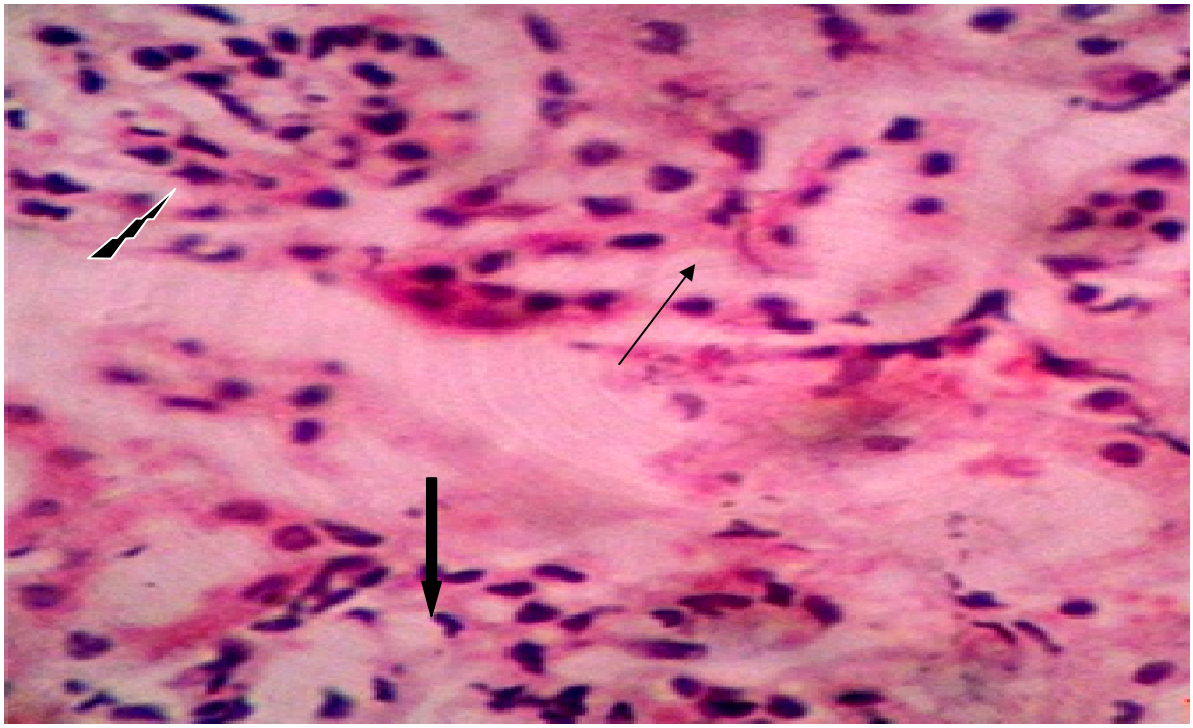


Plate IX: Section of kidney of rat dosed with lead describing the distal convoluted tubule (block arrows) and proximal convulated tubule (line arrows) and the glomeruli (lighting bolts), but there is loss of normal architecture of the glomeruli leading to near total disappearance of the glomeruli. H & E × 400

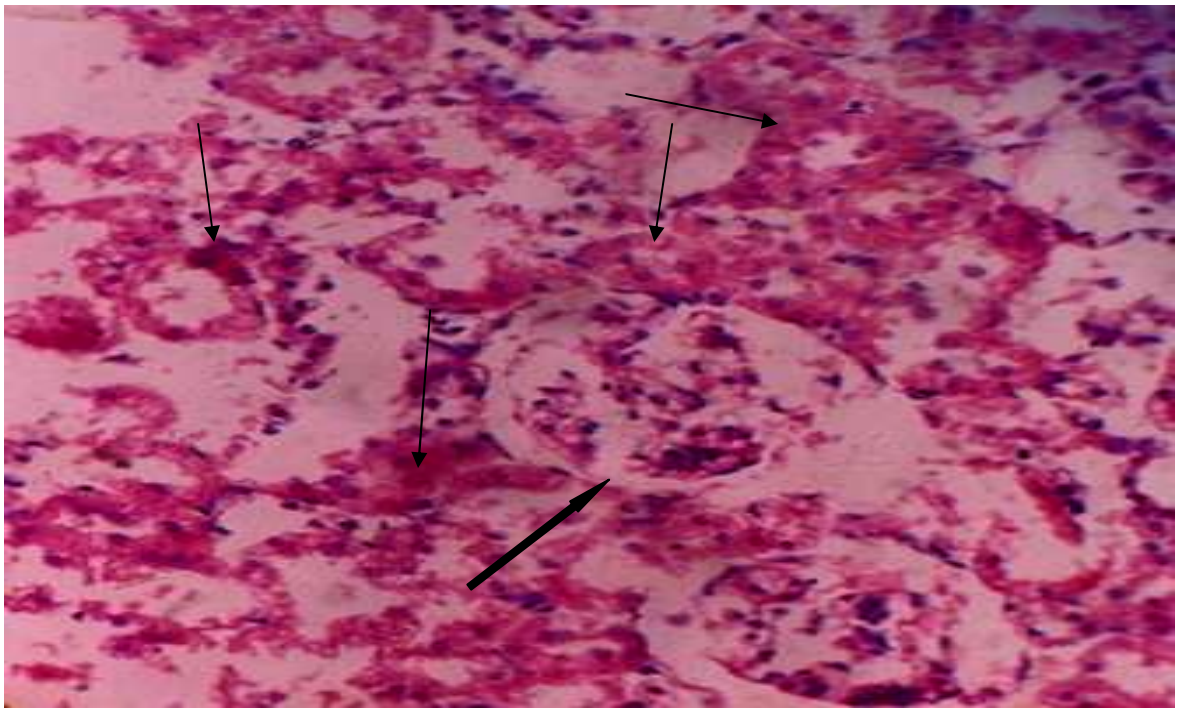


Plate X: Section of kidney of rat pre-treated with ascorbic acid prior to administration of lead acetate showing diffuse haemorrhages on the cortical area (line arrows) and glomeruli (block arrow). H & E × 400

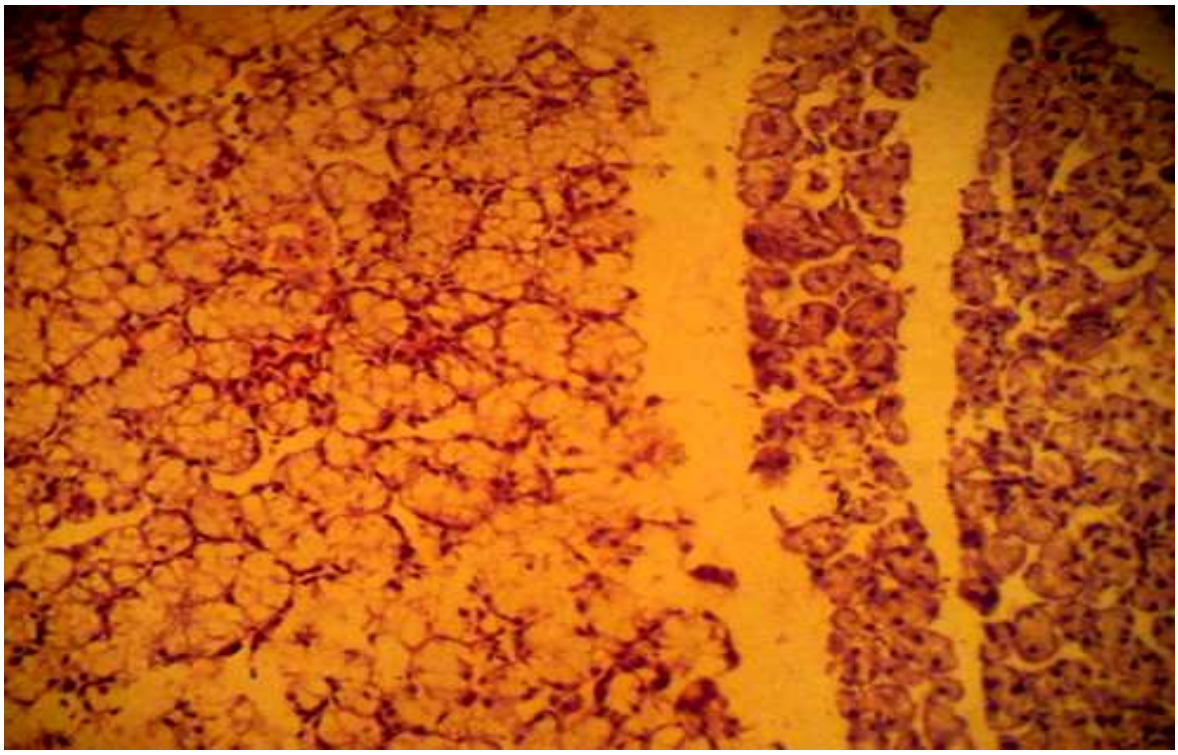


Plate XI: Section of thyroid gland of rat administered distilled water showing normal gland architecture with no apparent lesion. H & E × 100

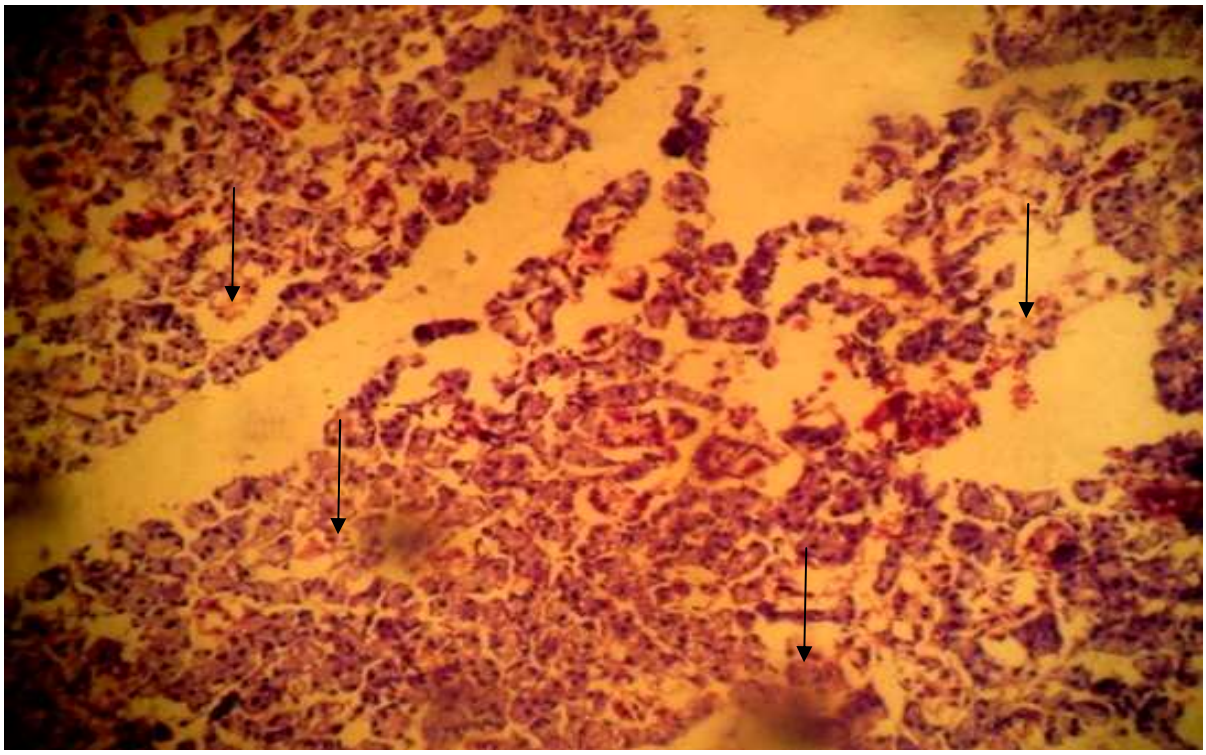


Plate XII: Section of thyroid of rats exposed to lead only, showing diffused haemorrhagic foci (line arrow). H & E × 100

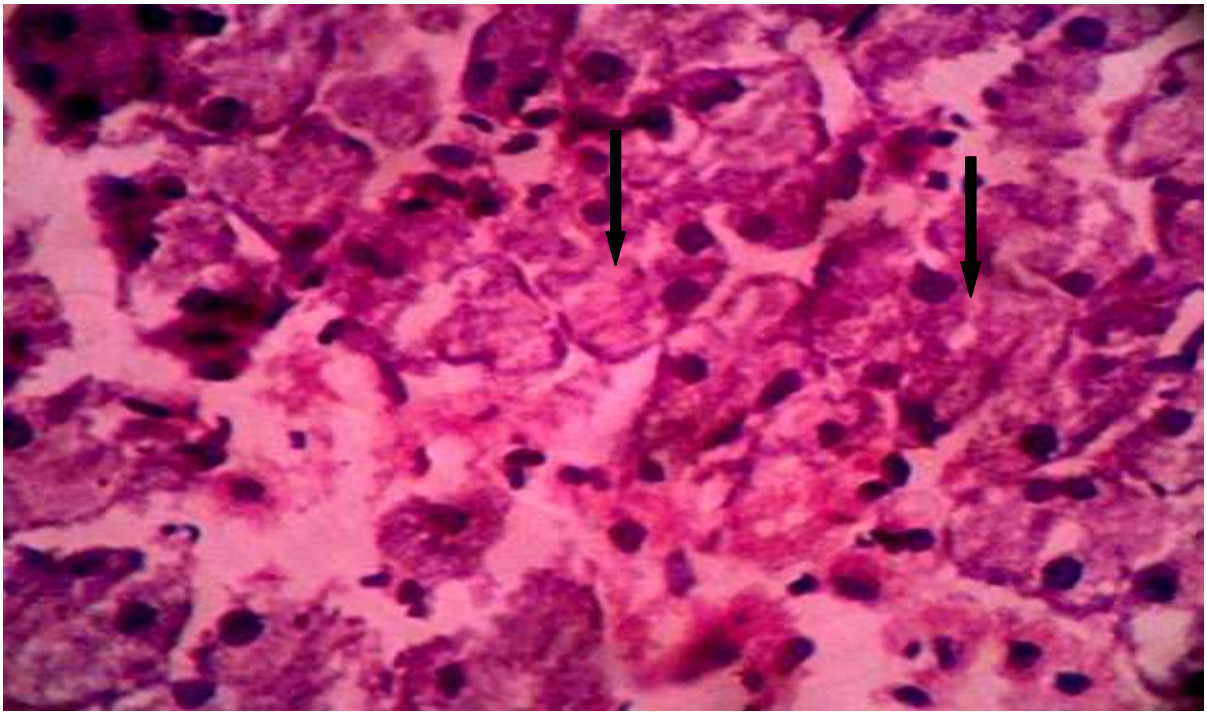


Plate XIII: Section of thyroid gland of rats pretreated with ascorbic acid prior to exposure to lead showing hyperplasia of secretory ducts (block arrows). H & E × 400

CHAPTER FIVE

5.0 DISCUSSION

The results of the present study showed that sub-chronic exposure of Wistar rats to lead acetate had no significant effect on body weight changes and did not cause overt signs of toxicity. This agrees with the findings of similar work done by Azzaoui *et al*. (2009) on lead in rats. The results obtained in the present study disagreed with that of Erdogan *et al*. (2005), who obtained a significant reduction in growth as evidenced by a decrease in body weight in broiler chickens following sub-chronic exposure to lead at the dose of 200 mg/kg. However, no significant weight changes were obtained in the present study even at the dose of 250 mg/kg. The difference in the results may be due to species variation and thus requires further investigation.

The fact that sub-chronic exposure of Wistar rats to lead had no significant ($P > 0.05$) effect on locomotor activity in the present study agrees with the findings of de Souza Lisboa *et al.* (2005); but disagrees with the finding of Nehru and Sidhu (2001), who demonstrated alterations in the locomotor activity of Wistar rats following oral exposure for 12 weeks to graded doses of lead, ranging from 10-200 mg/kg. This difference may be attributed to the longer duration of exposure (12 weeks) to lead in study conducted by Nehru and Sidhu (2001).

It has been shown that an increased frequency of rearing is indicative of a normal animal, which is very familiar with its environment and has good habituation learning and locomotor activity (Zhu *et al.*, 2001). Thus, the results of the study showed that rats pre-treated with lead had the least frequency of rearing, when compared with the control animals. In the absence of locomotor alterations, increased exploration as indicated by increased rearing is interpreted as anxiolytic effects, whereas decreased exploration as anxiogenic

effect. Thus, exposure of rats to lead resulted in anxiogenic effects. This finding is in agreement with the results of previous studies that established the anxiogenic effect of lead (Moreira *et al*., 2001; Leret *et al*., 2003; Jaako-Movits *et al*., 2005; Nieto-Fernandez *et al*., 2006; Soeiro *et al*., 2007). Although the mechanism of action of lead was not elucidated in the present study, several mechanisms have been suggested for the anxiogenic effect of lead. They include the involvement of increased activity of the hypothalamo-pituitary-adrenal axis, and alterations in dopaminergic and serotonergic neurotransmission in the hypothalamus (Moreira *et al*., 2001; Leret *et al*., 2003). The fact that the pre-treatment of rats with ascorbic acid only resulted in the highest frequency of rearing, when compared with the control animals, showed that a prior administration of ascorbic acid ameliorated the anxiogenic effect induced by lead.

It has been shown that an increase in the frequency of stretch-attend posture is indicative of increased anxiety (Brown *et al.,* 2007). Thus, in the present study, the exposure of Wistar rats to repeated low-dose lead resulted in an increase in stretch-attend postures, demonstrating an anxiogenic effect. The result of the stretch-attend posture further buttressed the fact that lead produces an anxiogenic effect. This effect was, however, found to be ameliorated by pre-treatment with ascorbic acid.

An increased frequency of defaecation and urination is indicative of increased anxiety (Brown *et al.,* 2007). The frequency of defaecation and urination in the open-field apparatus was higher in the lead-treated rats, when compared with that obtained in the control and ascorbic acid-treated groups. This again shows that ascorbic acid ameliorated the anxiogenic effect caused by lead in the rats.

The fact that the results on inclined plane apparatus showed no significant ($P > 0.05$) effect demonstrated that lead toxicity does not impair the coordination of neuromuscular

activity of rats; and, therefore, the pre-treatment of the exposed animals with ascorbic acid gave no beneficial effect. Similarly, the results of the present study showed that lead did not impair the elicitation of sensorimotor reflex in Wistar rats, as evidenced by the insignificant increase in excitability scores and non-beneficial effect of pre-treatment with ascorbic acid.

The current results on learning and short-term memory demonstrated a decrease in the cognitive function of rats exposed to repeated low-dose lead, indicating a reduction in the index of learning acquisition and short-term memory retention of the experimental animals. Several studies have demonstrated the effect of lead on learning and short-term memory in animals (Aldridge *et al*., 2005; Haider *et al*., 2005; Azzaoui *et al*., 2009) Although the poor memory retention of lead-exposed rats was not significant ($P > 0.05$), the exposed rats spent less time on the platform in the step-down avoidance inhibitory apparatus, compared to rats in other treatment groups. This fact, apparently, showed that lead impairs cognitive function in rats.

Several mechanisms of altered cognitive function following lead exposure have been proposed. One is the alterations in glutamate release from the hippocampus (Wisden *et al.*, 2000). The hippocampus is a brain area which is rich in glutamate receptors. Appropriate glutamate activity is critical for the development of the central nervous system, including the ontogeny of learning and memory (Adams *et al*., 2009). The effects of glutamate are often mediated through the N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid (AMPA) and kainate receptors (Frerking *et al*., 2001; Bortolotto *et al*., 2005; Adams *et al*., 2009). Infact, NMDA receptors are important in their role in long-term potentiation (Rison and Stanton, 1995; MacDonald *et al.,* 2006), which is a form of synaptic plasticity often used as a model system for the study of cognitive functions (Porterfield and Hendrich, 1993; Laroche *et al.*, 2000; O'Mara *et al.*, 2000). The learning process is based on the creation and remodelling of synapses and the toxic effect of lead on

this process suggests that lead specifically damages synaptic functions (Bradbury and Deane, 1993; Lasley and Gilbert, 2000; Bouton *et al*., 2001). Damage to the hippocampus has been reported to cause severe impairments in learning and memory (Eichenbaum *et al*., 1990; McDonald and White, 1995; Grigorenko *et al*., 1997), but this damage was found not to affect spatial information (Gilbert *et al.*, 2005).

Another mechanism is via decrements in the activities of acetylcholinesterase. Sub-chronic exposure to lead has resulted in a decrease in activity of acetylcholinesterase in rats (Nehru and Sidhu, 2002; Reddy *et al.*, 2003; Saxena and Flora, 2004). Similarly, the central cholinergic system has been linked to synaptic plasticity, learning and memory processes in several studies (Sachdev *et al*., 1998; Reddy *et al.*, 2007). A decrease in brain serotonin (5-hydroxytryptamine) levels has also been implicated as a cause of impaired cognitive function in lead-treated rats (Haider *et al*., 2005). In addition, rat brain neurotoxicity has been demonstrated to occur via the induction of oxidative stress as a result of the accumulation of delta-aminolevulinic acid during lead exposure (Tandon *et al*., 2002; Patrick, 2006; Wang *et al*., 2006). The increased lipoperoxidative changes in the brain and degenerative changes in the neurones observed in the lead-treated group shows that lead may have impaired learning and memory, partly due to oxidative damage.

The result of the present study demonstrated a beneficial effect of pre-treatment with ascorbic acid, as learning and short-term memory of lead-exposed rats were improved. Parle and Dhingra (2003) and Shahidi *et al.* (2008) demonstrated increases in memory and learning following ascorbic acid administration in mice and rats, respectively. The result is in agreement with that of Han *et al.* (2007), who reported that ascorbic acid may have potential benefits in treating lead-induced brain injury in the developing rat brain. Ascorbic acid, as a water-soluble antioxidant, has been used under several clinical conditions. It has a high toxicity threshold and its wide therapeutic window enhances its utility. It is able to penetrate

efficiently the blood-brain barrier (Frei and England, 1989; Schreiber and Trojan, 1991; Rose and Bote, 1993; Pilidori *et al*., 2001). The blood-brain barrier is crucial in protecting the central nervous system against toxic insults (Fazakas *et al*., 2005), and lead is known to damage the blood-brain barrier (Goldstein *et al*., 1974). In addition, agents that inhibit generation of reactive oxygen species in the brain may block tissue damage resulting from excitotoxicity from glutamate and loss of cellular calcium (Bose *et al.,* 1992; Siesjo, 1993). This mechanism involved in the pathogenesis of neuronal damage resulting in impaired cognition, further confirms the beneficial role of ascorbic acid in improving learning and memory in rats. The remediation of lead-induced brain lipoperoxidative changes and neuronal degeneration by ascorbic acid in this study supports the role of oxidative stress in lead-induced impairment of cognition.

The present findings on sex variation in cognitive function showed that lead-exposed female Wistar rats tend to have a better short-term memory than the males, but no difference ($P > 0.05$) in learning between the sexes. The better cognitive function of females obtained in the present study is in agreement with previous work done by Uysal *et al*. (2005, 2008), and may be attributed to the additional antioxidant effect of oestrogen, which is more in females than in males. This finding agrees with the previous results that the female gonadal hormone protects against several neurodegenerative diseases and cerebral ischaemia via various mechanisms (Oge *et al.,* 2003). The possible protective effects of oestrogen are mediated mainly by three ways; the activation of steroid receptors and/or modulation of a neurotransmitter and/or direct anti-oxidative action (Sartori-Valinotti *et al*., 2007). Oestrogen actions as antioxidants are derived probably from the presence of the phenolic A-ring in the structure of these molecules (Gilgun-Sherki *et al*., 2002; Prokai *et al*., 2005). Estrogens also have strong and positive influence by increasing the energy production efficiency of mitochondria. Thus, the hormone inhibits mitochondrial production of free-radical oxygen

molecules (Dantas *et al*., 2002). Since oxidative stress has been implicated in the lead-induced cognitive dysfunction, the administration of ascorbic acid in female rats may act to provide a pool of antioxidants needed to scavenge the reactive oxygen species generated following exposure to lead. Thus, there was an increase in the memory response of the female experimental animals pre-treated with ascorbic acid in the present study.

Several reports on the effect of lead on haematological parameters in humans and animals have been well documented. In the current study, haematological parameters of lead-exposed Wistar rats were significantly ($P < 0.05$) affected. The PCV, Hb concentration and total erythrocyte counts were decreased. The findings from this study are in agreement with the results obtained by other authors (Flora and Seth, 2000; Wang *et al*., 2007; Katavolos *et al*., 2008). The decrease observed in the PCV, Hb and total erythrocyte counts in the lead-exposed rats may be due to increased lipid peroxidation in erythrocyte cell membranes and subsequent haemoglobin oxidation (Ribarov and Benov, 1981; Saxena and Flora, 2004). Lead has been shown to bind to enzymes that have functional sulfhydryl groups, thereby rendering them non-functional. Delta-aminolevulinic acid dehydratase (ALAD) is a specific sulfhydryl-containing enzyme that is inhibited by lead (Gurer-Orhan *et al.,* 2004; Ahamed *et al*., 2005). ALAD is a critical enzyme in lead toxicity because the inhibition of ALAD lowers haeme production and increases levels of substrate delta-aminolevulnic acid. An elevated level of delta-aminolevulnic acid is known to stimulate reactive oxygen species production (Bechara, 1996). Pretreatment with ascorbic acid was able to ameliorate ($P < 0.05$) the decrease. Ascorbic acid is a water-soluble antioxidant that is found throughout the body as the ascorbate anion. It inhibits peroxidation of membrane phospholipids and acts as a scavenger of free radicals (Rice and Barone, 2000). Perhaps, another mechanism of action of ascorbic acid in ameliorating the decrease is via an increase in blood ALAD activity following its inhibition by lead in rats, as demonstrated by Flora *et al.* (2003).

The present study also revealed that repeated exposure of Wistar rats to low-level of lead may affect the immune cells. The total white blood cell counts increased significantly in lead-exposed Wistar rats, predominantly due to an increase in neutrophils. In addition, the amount of circulating lymphocytes decreased in lead-exposed animals. This further shows that lead plays a role in the homeostasis of the immune system. Leucocytosis due to neutrophilia with a corresponding lymphocytosis is indicative of immunosuppression. Evidence that lead induces immunosuppression has been reported (Gao *et al.,* 2006), and oxidative stress has been implicated in lead-induced immunosuppression (Ercal *et al.,* 2000). The findings agree with that of Nakhostin-Roohi *et al.* (2008), who obtained an increase in lymphocyte counts following ascorbic acid supplementation. In fact, Ströhle and Hahn (2009) reported that several cells of the immune system accumulate vitamin C and this is needed to perform their tasks and enhance their activities. Thus, the pretreatment of rats in the group exposed to lead with ascorbic acid ameliorated the adverse effects. Ascorbic acid has also been shown to stimulate peripheral lymphocyte proliferation and immunoglobulin productions (Tanaka *et al.,* 1994; Wintergerst *et al.,* 2006; Maggini *et al.,* 2007). This finding also further confirms that oxidative stress plays an important role in the immunosuppression due to lead exposure.

Glutathione is a cysteine-based molecule that is produced in the interior compartment of the lymphocytes (Meister and Anderson, 1983). In addition to acting as an important antioxidant for quenching free-radicals, the sulfhydryl complex of glutathione also directly binds to toxic metals, such as lead, that have a high affinity for sulfhydryl groups (Patrick, 2006). Lead may deplete the antioxidant reserve of the body either by directly acting on the lipid membranes of the lymphocytes or binding to the thiol group of glutathione, thus depleting its body reserve, and thereby resulting in a compensatory effect by lymphocytes. Therefore, it can be inferred that the depletion of circulating lymphocytes obtained in the

present study may be a direct result of the effect of lead on circulating lymphocytes and glutathione. It has been established that the lymphocytes tend to produce more glutathione to compensate for the loss and in the process they "burn out", and this leads to their depletion (Garcon *et al*., 2004; Ahamed *et al*., 2005; Patrick, 2006).

The fact that there was no effect on circulating eosinophils and basophils in the rats exposed to lead, apparently, implies that inflammatory response is not possible sequelae of lead-induced toxicity at low doses. Similarly, no significant effect was observed in the values of monocytes of the rats, except those treated with ascorbic acid only that had an increase ($P < 0.05$) in circulating monocytes. This suggests that ascorbic acid may boost the activity of monocytes. The mechanism for this activity is, however, not well understood.

The increase in neutrophil/lymphocyte ratio observed in the present study is in agreement with the findings of Minka and Ayo (2007) and Minka *et al.* (2009) that the ratio increased in stress situations, especially in those induced via free-radical mechanism. Thus, the administration of ascorbic acid decreased the ratio in the present study. This finding confirms the previous result obtained by Ayo *et al.,* (2006) and Minka *et al.,* (2009), that ascorbic acid ameliorates the risk of adverse effects due to free-radical induced cell damages and destruction.

The present study demonstrated an increase in erythrocyte osmotic fragility following sub-chronic lead exposure in rats, apparently due to increased oxidative damage resulting in the impairment of erythrocyte membrane integrity and haemolysis (Stern, 1986; Wagner *et al.,* 1998). The finding of the present study agree with that of Quintana-Escorza *et al.* (2007), who reported an increase in osmotic fragility of erythrocytes in lead-exposed workers. The erythrocyte has been demonstrated to be a good model for the investigation of oxidative stress (Chihuailaf *et al*., 2002). Decreased osmotic resistance of the erythrocyte membranes

between 0.5 % and 0.7 % NaCl concentrations may be due to the peroxidation of the unsaturated double bonds of the membrane lipids (Brzezinska-Slebodzinska, 2003). Erythrocytes have also been shown to be the route of lead distribution to organs and tissues due to its high affinity for the metal, thereby decreasing the cell membrane fluidity and increasing the rate of erythrocyte haemolysis resulting from ROS-induced lipid peroxidation in the RBC membranes (Lawton and Donaldson, 1991; Quintana-Escorza *et al.,* 2007). Similarly, it has been shown that the erythrocytes of lead-exposed individuals are more fragile than those obtained from normal subjects, partly due to the inhibition of ALAD, an enzyme required for haeme synthesis, and the effect of free-radicals generated as a result of the accumulation of delta-aminolevulinic acid (Stoleski *et al.,* 2008). Again, the fact that pre-treatment of rats with ascorbic acid in this study increased the osmotic resistance of the erythrocyte membrane between 0.5 % and 0.7 % NaCl concentration, further confirms the role of oxidative stress in the pathophysiology of low-dose lead poisoning. The findings of this study which agree with previous results obtained by other authors (May *et al*., 1998; Mendiratta *et al.*, 1998; Ross *et al*., 1999) demonstrated, for the first time, the protective effect of ascorbic acid on the erythrocytes of rats repeatedly exposed to lead.

The results of the present study demonstrated that lead at 250 mg/kg/day did not exert any effect on the activities of ALT and ALP, although it decreased the activities of AST and LDH. Erdogan *et al*. (2005) and Khan *et al*. (1994) have shown also that lead had no significant effects on LDH, ALT and AST activities in broiler chickens, exposed to 200 mg/kg daily for 8 weeks. The findings disagree with that of Khan *et al*. (1993), who demonstrated an increase in AST activity. The differences in the results are not well understood. However, it may be due to differences in duration of exposure. Changes in AST and LDH activity is less specific for liver disease, they may also signal damages to the cardiac and skeletal muscles, RBC, kidney and brain tissue (Rochling, 2001). The

insignificant ($P > 0.05$) effect of lead on ALT and ALP observed in this study may be due to the relatively short duration and low intensity of exposure. Pre-treatment of the rats with ascorbic acid elevated the concentrations of the depleted enzymes. Microscopic examination of the liver of the group pretreated with ascorbic acid revealed milder lesions, demonstrating some protection by ascorbic acid. Therefore, it may be inferred that oxidative stress plays a significant role in lead-induced liver damage and that pretreatment with ascorbic acid ameliorated the damage. Ascorbic acid has been shown to ameliorate damage to hepatocytes from oxidative stress induced by lead (Wang *et al.*, 2007).

Repeated exposure to lead has been shown in the present study to inhibit the activities of creatine kinase (CK). This finding is in agreement with the result obtained by Zhou *et al.* (2002). The alteration in CK may partly underlie the neurotoxicity of lead observed in the present study. Creatine kinase exists as two isozymes, CK-M which is a muscle leakage enzyme and CK-B which is expressed mainly by the brain and spinal cord (Manos *et al.*, 1991; Wang *et al.*, 2002). Similarly, decreased activity of CK in this study may also be an indication of liver damage, since individuals suffering from alcoholic liver disease were found to have a low CK activity (Wang *et al*., 2002). It is known that lead binds to enzymes that have functional sulfhydryl (SH-) groups, thus rendering them non-functional (Patrick, 2006). CK has a SH-base functional site and the behaviour of its enzymatic activity might represent other functional proteins with SH-base (Zhou and Tsou, 1987). Neurotoxic agents such as acrylamide, ethylene oxide and 1-bromopropane were shown to suppress CK activity in the brain and blood of rats (Korhriyama *et al.*, 1994; Matsuoka, 1996; Wang *et al.*, 2002). Pretreatment with ascorbic acid increased the activity of CK. Evans (2000) and Nakhostin-Roohi *et al.* (2008) have shown that CK activity increased following ascorbic acid administration. The amelioration of lead-induced alteration in CK activity by ascorbic acid may be due to restoration of liver and brain cytoarchitecture.

The present study showed that serum total protein value in lead decreased significantly when compared to the control. This finding confirms the previous results that lead impairs the activity of protein enzymes, thus reducing the circulating protein level (Chang *et al.,* 2006; Ashry *et al.,* 2009). Metal ions such as Pb^{2+} bind to protein or polypeptides. In addition, oxidation of protein side chains by ROS may also result in protein depletion (Stadtman, 1992; Dalle-Donne *et al.,* 2005; Sayre *et al.,* 2008). The decrease in total protein concentration may be due to the slight, but insignificant ($P > 0.05$) decrease in albumin concentration in the lead exposed animals observed in this study. Again, this agrees with the findings of Ashry *et al.* (2009). Erdogan *et al.* (2005) however demonstrated that lead had no effect on albumin and total protein following lead toxicity in broilers chickens, which contravenes the results of the present study. The difference in the result may be due to species difference and the duration of exposure. Alterations in the concentrations of total protein and albumin in the rats exposed to lead only may be due to the hepatic and glomeruli changes observed under microscopic examination of the liver and kidney.

The result of the present study also showed that pre-treatment of lead-exposed rats with ascorbic acid increased the concentration of total protein, apparently as a result of the increase in globulin value. Campbell *et al.* (1999) and Naidu (2003) showed that ascorbic acid stimulates the immune system by enhancing T-cell proliferation which ultimately induces the B-cells to synthesize immunoglobulin, especially during infection. This finding agrees with the results of the present study on the effect of ascorbic acid on circulating lymphocytes and those reported by other authors (Wintergerst *et al*., 2006; Maggini *et al*., 2007). Lipid peroxidation products of PUFA have been shown to cause protein degradation and denaturation, thereby contributing to low serum protein (Chiba and Iwata, 2002). The fact that pre-treatment with ascorbic acid improved the total protein concentration and

restored the liver and kidney cytoarchitecture further confirms the role of lipid peroxidation in the mechanism of lead-induced hypoproteinaemia observed in this study.

The slight but insignificant ($P > 0.05$) increase in creatinine, urea and uric acid concentrations in rats exposed to lead in this study may be due to the low-dose and shorter duration of exposure, 6 weeks. Prolonged exposure of individuals to lead has been associated with chronic renal failure (Ekong *et al.,* 2006). The marginal increase in serum concentration of urea in rats exposed to lead indicates some level of kidney damage, and elevation of uric acid has been associated with obstruction of kidney since uric acid is a product of purine metabolism, which must be excreted by the kidney (Ambali, 2009). An elevated creatinine has been shown to be associated with renal insufficiency and renal obstruction (Ambali *et al.,* 2007). The histopathological lesions in the kidney glomeruli may be responsible for the marginal increase in the serum levels of uric acid, urea and creatinine observed in this study. This is in agreement with the findings of McMurry *et al.* (1995). The present study also showed a less severe degree of degenerative changes in the kidneys of rats pretreated with ascorbic acid, which may have contributed to the apparent restoration of uric acid, urea and creatinine concentrations.

The results of the present study also showed an increase in MDA concentration in the brain of lead-exposed rats. MDA is a reliable index of lipid peroxidation and has been shown to correlate directly with oxidative stress (Xu *et al.,* 2008; Gupta *et al.,* 2009; Lodovici *et al.,* 2009). The brain is particularly vulnerable to oxidative insult because of its high rate of oxygen utilisation, the relatively poor concentrations of classical antioxidants and related enzymes, and the high content of polyunsaturated lipids (Adonaylo and Oteiza, 1999; Sayre *et al.,* 2008). Lead alters the functional integrity of the blood-brain barrier, thus facilitating its entry into the brain (Goldstein *et al*., 1974). The lipid peroxidation process results mainly from the weak bis-allylic C-H bond present in polyunsaturated lipids and oxidative

phosphorylation of mitochondria in the brain (Sayre *et al.,* 2008). The findings of the present study agree with those of Wang *et al*. (2006), and Candan and Tuzmen (2008) that lead increase the brain concentration of MDA. The increased lipid peroxidation may have been responsible for the necrotic lesions, possibly through the induction of apoptosis, as evidenced by the loss of neurones due to karyohexes and pyknosis. These findings agree with those of Krametter-Froetscher *et al.* (2007).

The fact that the pre-treatment of lead-exposed rat with ascorbic acid decreased the MDA concentration further confirms the role of oxidative stress in the development of neurotoxicity following low-level lead exposure. Again, the findings of this study agree with the findings of Han *et al.* (2007) and Antonio-Garcia and Masso-Gonzalez (2008), who demonstrated the beneficial role of ascorbic acid in the brain of lead-exposed rats during brain development. The decrease in brain MDA concentration and the histopathologic cytoarchitecture observed in rats pretreated with ascorbic acid further confirms the role of oxidative stress as a mechanism of lead-induced neurotoxicity. Ascorbic acid has the ability to scavenge ROS by donating free electrons, thereby preventing oxidative damage to important biological macromolecules such as DNA, proteins and lipids (Carr and Frei, 1999). Several authors have reported that antioxidants mitigate oxidative stress induced by lead (Hsu *et al.,* 1998; Patra *et al.,* 2001; Attri *et al.,* 2003).

The effect of repeated low-level lead exposure on thyroid function was investigated since the disruption of some endocrine systems; especially thyroid hormones may result in a decrease in neurobehavioural activities. Several xenobiotics have been reported to alter thyroid function (Porterfield and Hendrich, 1993). Lead blocks the process of long-term potentiation, a form of synaptic plasticity that has often been used as a model system for the study of cognitive potential (Porterfield and Hendrich, 1993; Niemi *et al.,* 1996; Hussain *et al.,* 2000; Nguyen *et al.,* 2000). Experimental hypothyroidism has been shown to reduce

long-term potentiation (Niemi *et al.*, 1996) and alters synaptic plasticity in developmental hypothyroidism (Gilbert, 2004). A slight decrease ($P > 0.05$) in serum T_3 and T_4, and a corresponding increase in TSH concentrations were observed following lead exposure. The decrease in thyroid hormones in group exposed to lead only may be due to a central depression of the thyroid axis, an alteration in thyroxine metabolism or binding to proteins (Robins *et al.,* 1983).

Mendes-Aquiar *et al.* (2008) showed that T_3 regulates extracellular glutamate levels by modulating the astrocytic glutamate transporters; and that it improves the astrocytic environment, thereby promoting neural development and neuroprotection. In addition, astrocytic glutamate transporters have been implicated in lead toxicity (Struzynska, 2009). The cognitive deficits observed in lead-exposed group may have been partly due to alteration in the levels of T_3 and T_4. The findings in this study also supports previous reports that hypothesized that decreased T_4 may be a relevant predictor of long lasting developmental neurotoxicity (Axelstad *et al.,* 2008). Hypothyroidism has also been associated with oxidative stress in the brain of rats (Ali and Davydov, 2007) and increased erythrocyte osmotic fragility (Dariyerli *et al.,* 2004), a feature observed in the present study in rats exposed to lead only. Haemorrhagic lesions observed in rats exposed to lead only, may be sequelae of oxidative damage to the capillaries of the thyroid follicles.

The results obtained in the present study agree with the findings of Piao *et al.* (2007). Several authors, however, are of the opinion that hypothyroidism may not be sequelae of occupational lead exposure in humans (Refowitz, 1984; Siegal *et al.,* 1989; Schumacher *et al.,* 1998). However, some authors (Robins *et al.,* 1983; Bielecka *et al.,* 1987; Tuppurainen *et al.,* 1988; Lasisz *et al.,* 1992) reported that thyroid function might be depressed as a result of intense long-term lead exposure. Heavy occupational exposure to lead, sufficient to cause clinical poisoning, has been associated with disturbances in thyroid function in men (Cullun

et al., 1984). The result of the present study also showed that pretreatment with ascorbic acid improved the thyroid function. Experimental transient and persistent hypothyroidism has been shown to alter antioxidant defense systems of rats thereby, modulating vital body functions in this specie (Sahoo and Chainy, 2007; Sahoo *et al.,* 2008). It appears that the ameliorative effect of ascorbic acid on thyroid function was mediated via its anti-oxidative action. Gupta and Kar (1998) and Deshpande *et al.* (2002) have reported the amelioration of thyroid dysfunction following exposure to cadmium and methimazole, respectively. The hyperplasia of the acinar observed following microscopic examination of the thyroid gland may be a stimulatory response to secrete T_4, probably due to protection offered by ascorbic acid from damage to the thyroid gland.

This research has further confirmed the role of oxidative stress in the pathophysiology of low-level lead poisoning. The ameliorative effects of ascorbic acid on neurobehavioural, cognitive, haematological, biochemical and tissue pathological changes in rats further lends credence to the oxidative stress mechanism of lead poisoning. For the first time, this study has shown that ascorbic acid is able to ameliorate decrease in learning ability and short-time memory loss due to repeated exposure to lead. Also, for the first time, we have also demonstrated that ascorbic acid has beneficial effects on the haemotoxicity, and biochemical and tissue pathological changes following repeated exposure to lead in rats, as evidenced by the amelioration of these changes.

SUMMARY, CONCLUSION AND RECOMMENDATION

Summary

The present study has shown that sub-chronic exposure of adult Wistar rats to lead caused:

1. An increase in anxiety and impairment in cognition (learning and memory), with the females having better short-term memory than the males.
2. A reduction in haematological parameters such as packed cell volume, haemoglobin concentration and total erythrocyte counts. It also caused leucocytosis due to marked neutrophilia and lymphopaenia.
3. A slight increase in erythrocyte osmotic fragility which made the red blood cells susceptible to lysis.
4. Decreased activity of some serum enzymes such as aspartate aminotransferase, lactate dehydrogenase and creatine kinase, which indicates some level of hepatic and kidney damage.
5. A decrease in the level of total protein apparently due to slight decrease in albumin concentration.
6. Increased lipid peroxidation in the brain evidenced by increased thiobarbituric reactive substances, malondialdehyde in this organ. This shows that lipid peroxidation and oxidative stress plays an important role in the neurobehavioural and biochemical changes observed in rats exposed to lead only.
7. An alteration in thyroid function characterised by reduction in the level of $T_{3,}$ T_4 and slight increase in TSH; thus suggestive of damage to the thyroid and/or the hypothalamus or pituitary glands.

The study has also shown for the first time that pre-treatment with antioxidant ascorbic acid improved deficits in neurobehavioural and cognitive changes induced by sub-chronic

exposure to lead. In addition, antioxidant ascorbic acid ameliorated lead-induced deficits in PCV, Hb, RBC and WBC. Furthermore, the study has for the first time revealed the ability of ascorbic acid to protect the integrity of RBC membrane as observed in amelioration of the lead-induced increase in erythrocyte osmotic fragility. Similarly, pre-treatment with ascorbic acid protect against lead-induced hypoproteinaemia. The study has also for the first time shown the ability of ascorbic acid to ameliorate decreases in the activity of serum AST, LDH and creatine kinase resulting from low-level lead exposure. The study has further reaffirmed the ability of ascorbic acid to protect the body against lead-induced lipid peroxidation as demonstrated by reduced concentration of MDA in the vitamin pre-treated groups.

Conclusions

The conclusion, the study has shown that;

1. Sub-chronic low-dose lead exposure to Wistar rats caused significant alteration in neurobehavioural, cognitive, haematological, biochemical and tissue pathological changes.
2. Lipid peroxidation is an important molecular mechanism involved in neurobehavioural and cognitive changes in sub-chronic low-dose lead exposure in rats.
3. Pre-treatment with ascorbic acid ameliorated lead-induced neurotoxic, haematological, biochemical and tissue pathological changes in rats.

Recommendations

Specific recommendations

1. Further work should be done to determine the relationship between neurotransmitters, acetylcholinesterase and delta-aminolevulinic acid and their response to ascorbic acid.

2. Other markers of oxidative stress such as glutathione, glutathione peroxidise, superoxide dismutase, catalase, 8-hydroxydeoxyguanosine and caspase-3 should be measured in the serum and tissues, in other to establish their relationship with cognitive, haematological, biochemical and tissue pathological changes following lead exposure and response to ascorbic acid pretreatment.
3. Region specific studies of the brain should be conducted to establish areas most susceptible to low-level lead exposure and response to pretreatment with ascorbic acid.

General recommendations

1. Reduction of exposure to sources of lead, rather than treatment should be emphasized in individuals at risk.
2. The use of leaded paints, plastics, pipes and gasoline should be discouraged.
3. High risk populations should be encouraged to supplement their diets with antioxidants such as ascorbic acid.
4. Due to preponderance of lead in the environment and their adverse effects, animals such as sniffer dogs, African giant rat, geese and ostriches, whose cognitive function is been put to task should be pretreated regularly with antioxidants such, ascorbic acid.

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APPENDIX I

Effects of sub-chronic lead exposure and pretreatment with ascorbic acid on body weight, memory and learning in Wistar rats

Parameters	**Treatment groups (Mean ± SEM)**			
	Control	**Lead (250 mg/kg)**	**Ascorbic acid (100 mg/kg)**	**Lead + ascorbic acid (250 mg/kg + 100 mg/kg)**
% Weight gain	103.88 ± 6.94^{a}	109.81 ± 2.68^{a}	112.12 ± 8.09^{a}	109.27 ± 7.73^{a}
Memory (s)	104.00 ± 8.58^{a}	71.70 ± 14.33^{a}	107.36 ± 8.27^{a}	94.71 ± 15.32^{a}
Memory (males)	120.00 ± 0.00^{a}	61.00 ± 17.63^{b}	107.00 ± 12.75^{a}	114.00 ± 5.67^{a}
Memory (females)	88.00 ± 0.20^{b}	82.40 ± 0.20^{a}	107.00 ± 0.50^{a}	80.00 ± 0.58^{b}
Learning (No. of foot-shocks)	1.20 ± 0.13^{b}	2.80 ± 0.33^{a}	1.75 ± 0.25^{b}	1.71 ± 0.29^{b}
Learning (males)	1.20 ± 0.20^{a}	2.80 ± 0.20^{a}	1.50 ± 0.50^{a}	2.00 ± 0.58^{a}
Learning (females)	1.20 ± 0.20^{a}	2.80 ± 0.66^{a}	2.00 ± 0.00^{a}	1.50 ± 0.29^{a}

$^{a, b}$ = Values with different superscript letters are significantly ($P < 0.05$) different, while those with the same superscript letters are not significant ($P > 0.05$).

APPENDIX II

Effect of sub-chronic lead exposure and pretreatment with ascorbic acid on open-field parameters in Wistar rats

Parameters	**Treatment groups (Mean ± SEM)**			
	Control	**Lead (250 mg/kg)**	**Ascorbic acid (100 mg/kg)**	**Lead + ascorbic acid (250 mg/kg + 100 mg/kg)**
Rearing	33.10 ± 8.61^{a}	30.00 ± 4.41^{a}	38.30 ± 8.49^{a}	22.40 ± 4.26^{a}
Stretch-attend posture	4.50 ± 0.70^{ab}	4.90 ± 1.05^{a}	4.20 ± 0.80^{ab}	2.50 ± 0.52^{b}
Urination	2.20 ± 0.74^{a}	2.80 ± 0.73^{a}	1.80 ± 0.55^{a}	2.80 ± 0.63^{a}
Defaecation	19.30 ± 2.21^{bc}	24.10 ± 1.46^{ab}	14.30 ± 2.24^{c}	24.20 ± 2.60^{a}
Locomotor activity	39.90 ± 12.67^{a}	43.60 ± 6.47^{a}	42.10 ± 15.97^{a}	21.70 ± 5.52^{a}

$^{a, b}$ = Values with different superscript letters are significantly ($P < 0.05$) different, while those with the same superscript letters are not significant ($P > 0.05$).

APPENDIX III

Effects of repeated exposure to lead and pretreatment with ascorbic acid on frequency of rearing in Wistar rats

Time	**Treatment groups (Mean ± SEM)**			
	Control	**Lead (250 mg/kg)**	**Ascorbic acid (100 mg/kg)**	**Lead + ascorbic acid (250 mg/kg + 100 mg/kg)**
Day 0	17.50 ± 4.48	11.40 ± 3.06	17.50 ± 3.08	12.60 ± 2.70
Day 2	6.90 ± 1.97	10.90 ± 3.14	6.40 ± 2.77	3.20 ± 1.36
Week 1	2.50 ± 1.17	3.50 ± 0.87	3.70 ± 1.63	2.00 ± 0.76
Week 2	2.60 ± 1.06	2.30 ± 0.83	7.56 ± 2.33	2.70 ± 1.51
Week 3	2.20 ± 0.96	1.20 ± 0.55	2.38 ± 0.68	1.38 ± 0.42
Week 6	1.40 ± 0.52	0.70 ± 0.21	2.50 ± 0.87	1.14 ± 0.40

APPENDIX IV

Effects of repeated exposure to lead and pretreatment with ascorbic acid on frequency of stretch-attend posture in Wistar rats

Time	**Treatment groups (Mean ± SEM)**			
	Control	**Lead (250 mg/kg)**	**Ascorbic acid (100 mg/kg)**	**Lead + ascorbic acid (250 mg/kg + 100 mg/kg)**
Day 0	1.60 ± 0.40	2.00 ± 0.80	0.90 ± 0.28	1.00 ± 0.29
Day 2	1.30 ± 0.47	1.20 ± 0.42	1.40 ± 0.43	0.50 ± 0.27
Week 1	0.40 ± 0.22	0.90 ± 0.41	1.00 ± 0.24	0.20 ± 0.20
Week 2	0.50 ± 0.31	0.40 ± 0.16	1.00 ± 0.37	0.60 ± 0.27
Week 3	0.40 ± 0.16	0.10 ± 0.10	0.00 ± 0.00	0.13 ± 0.13
Week 6	0.30 ± 0.15	0.30 ± 0.15	0.13 ± 0.13	0.14 ± 0.14

APPENDIX V

Effects of repeated exposure to lead and pretreatment with ascorbic acid on frequency of urination in Wistar rats

Time	Treatment groups (Mean ± SEM)			
	Control	**Lead (250 mg/kg)**	**Ascorbic acid (100 mg/kg)**	**Lead + ascorbic acid (250 mg/kg + 100 mg/kg)**
Day 0	0.40 ± 0.16	0.30 ± 0.15	0.50 ± 0.22	0.20 ± 0.13
Day 2	0.40 ± 0.16	0.40 ± 0.22	0.30 ± 0.21	0.30 ± 0.15
Week 1	0.40 ± 0.22	0.50 ± 0.17	0.50 ± 0.17	1.30 ± 0.34
Week 2	0.40 ± 0.16	0.50 ± 0.22	0.11 ± 0.11	0.50 ± 0.22
Week 3	0.30 ± 0.15	0.50 ± 0.17	0.38 ± 0.18	0.38 ± 0.18
Week 6	0.30 ± 0.15	0.60 ± 0.16	0.13 ± 0.13	0.43 ± 0.20

APPENDIX VI

Effects of repeated exposure to lead and pretreatment with ascorbic acid on frequency of defaecation in Wistar rats

Time	Treatment groups (Mean ± SEM)			
	Control	**Lead (250 mg/kg)**	**Ascorbic acid (100 mg/kg)**	**Lead + ascorbic acid (250 mg/kg + 100 mg/kg)**
Day 0	1.60 ± 0.56	3.50 ± 0.85	2.00 ± 0.52	3.40 ± 0.93
Day 2	3.20 ± 0.80	2.40 ± 0.75	2.90 ± 0.67	3.90 ± 0.55
Week 1	4.10 ± 0.75	4.50 ± 0.52	2.20 ± 0.51	5.10 ± 0.85
Week 2	1.50 ± 0.86	3.50 ± 0.87	1.00 ± 0.53	3.40 ± 0.62
Week 3	4.10 ± 0.74	5.30 ± 0.58	3.25 ± 0.88	5.25 ± 0.49
Week 6	4.80 ± 0.73	4.90 ± 0.53	4.63 ± 0.80	6.00 ± 0.62

APPENDIX VII

Effects of repeated exposure to lead and pretreatment with ascorbic acid on frequency of locomotor activity in Wistar rats

Time	**Treatment groups (Mean ± SEM)**			
	Control	**Lead (250 mg/kg)**	**Ascorbic acid (100 mg/kg)**	**Lead + ascorbic acid (250 mg/kg + 100 mg/kg)**
Day 0	16.60 ± 7.02	14.30 ± 5.00	19.70 ± 8.98	10.90 ± 4.43
Day 2	11.30 ± 5.13	14.30 ± 3.40	10.67 ± 7.58	4.80 ± 2.63
Week 1	3.10 ± 2.47	3.60 ± 2.00	2.33 ± 1.60	3.20 ± 1.77
Week 2	2.40 ± 1.07	4.30 ± 3.16	5.00 ± 3.53	1.90 ± 1.51
Week 3	5.40 ± 3.04	4.50 ± 2.20	3.00 ± 3.00	0.00 ± 0.00
Week 6	1.10 ± 0.90	2.60 ± 1.42	4.38 ± 2.55	1.29 ± 1.13

APPENDIX VIII

Effect of sub-chronic lead exposure and pretreatment with ascorbic acid on the performance of rats on the inclined plane apparatus

Time	**Treatment groups (Mean ± SEM)**			
	Control	**Lead (250 mg/kg)**	**Ascorbic acid (100 mg/kg)**	**Lead + ascorbic acid (250 mg/kg + 100 mg/kg)**
Week 1	55.50 ± 1.67	55.00 ± 1.29	52.00 ± 0.82	52.50 ± 0.83
Week 2	54.00 ± 1.25	57.50 ± 2.01	56.00 ± 1.25	56.50 ± 1.07
Week 3	60.00 ± 1.30	59.50 ± 1.38	59.00 ± 0.67	56.00 ± 1.00
Week 4	58.50 ± 1.30	54.00 ± 2.21	54.50 ± 1.67	55.50 ± 0.90

APPENDIX IX

Effect of sub-chronic lead exposure and pretreatment with ascorbic acid on the excitability scores of Wistar rats

Time	Treatment groups (Mean ± SEM)			
	Control	**Lead (250 mg/kg)**	**Ascorbic acid (100 mg/kg)**	**Lead + ascorbic acid (250 mg/kg + 100 mg/kg)**
Day 0	4.40 ± 0.27	4.30 ± 0.26	4.50 ± 0.27	4.40 ± 0.27
Day 2	4.60 ± 0.22	4.60 ± 0.22	4.80 ± 0.20	4.60 ± 0.27
Week 1	5.00 ± 0.00	4.80 ± 0.20	4.80 ± 0.20	4.90 ± 0.10
Week 2	5.00 ± 0.00	5.00 ± 0.00	4.90 ± 0.10	4.90 ± 0.10
Week 3	4.90 ± 0.10	5.00 ± 0.00	5.00 ± 0.00	4.90 ± 0.10
Week 6	4.80 ± 0.13	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00

APPENDIX X

Effects of treatments on cumulative inclined plane and excitability score of Wistar rats (Mean ± SEM)

Treatment groups	Cumulative inclined angle (°) (Mean ± SEM)	Cumulative excitability score (Mean ± SEM)
Control	57.00 ± 0.73[a]	4.78 ± 0.08[a]
Lead (250 mg/kg)	56.50 ± 1.10[a]	4.78 ± 0.10[a]
Ascorbic acid (100 mg/kg)	55.38 ± 0.62[a]	4.83 ± 0.12[a]
Lead + Ascorbic acid (250 mg/kg + 100 mg/kg)	55.13 ± 0.54[a]	4.78 ± 0.09[a]

[a] = Values with the same superscript letters are not significant (P > 0.05).

APPENDIX XI

Effects of sub-chronic lead exposure and pretreatment with ascorbic acid on haematological indices of Wistar rats

Parameters	**Treatment groups (Mean ± SEM)**			
	Control	**Lead (250 mg/kg)**	**Ascorbic acid (100 mg/kg)**	**Lead + ascorbic acid (250 mg/kg + 100 mg/kg)**
Haemoglobin concentration (g/dL)	14.35 ± 0.21^{ab}	13.13 ± 0.35^{c}	14.55 ± 0.26^{a}	13.66 ± 0.25^{bc}
Packed cell volume (%)	47.54 ± 1.02^{a}	44.03 ± 1.08^{b}	49.10 ± 1.09^{a}	47.70 ± 1.35^{a}
Total erythrocyte counts ($\times 10^{12}$/L)	8.03 ± 0.18^{a}	7.37 ± 0.17^{b}	8.41 ± 0.23^{a}	8.32 ± 0.30^{a}
Total leucocyte count ($\times 10^{6}$ g/dL)	5.21 ± 0.35^{b}	6.44 ± 0.50^{a}	4.98 ± 0.34^{b}	5.23 ± 0.23^{b}
Neutrophils (%)	17.20 ± 1.71^{ab}	22.40 ± 1.65^{a}	19.50 ± 1.51^{ab}	16.89 ± 2.04^{b}
Lymphocytes (%)	79.40 ± 2.11^{a}	73.50 ± 1.47^{b}	75.80 ± 1.96^{ab}	79.89 ± 2.00^{a}
Eosinophils (%)	3.10 ± 0.89^{a}	3.40 ± 0.78^{a}	2.50 ± 0.85^{a}	3.33 ± 0.37^{a}
Monocytes (%)	0.30 ± 0.21^{b}	0.60 ± 0.27^{b}	1.70 ± 0.47^{a}	0.22 ± 0.22^{b}
Basophils (%)	0.00 ± 0.00^{a}	0.10 ± 0.10^{a}	0.50 ± 0.22^{a}	0.22 ± 0.22^{a}
Neutrophil/Lymphocyte ratio	0.22 ± 0.03^{b}	0.31 ± 0.03^{a}	0.26 ± 0.02^{ab}	0.22 ± 0.03^{b}

$^{a, b}$ = Values with different superscript letters are significantly ($P < 0.05$) different, while those with the same superscript letters are not significant ($P > 0.05$).

APPENDIX XII

Effects of sub-chronic lead exposure and pretreatment with ascorbic acid on *in vitro* erythrocyte osmotic fragility of Wistar rats.

	Percent haemolysis (Mean ± SEM)			
% NaCl Concentration	**Control**	**Lead (250 mg/kg)**	**AA (100 mg/kg)**	**Lead + AA (250 mg/kg + 100 mg/kg)**
0	5.89 ± 0.52[a]	5.75 ± 0.63[a]	5.88 ± 0.47[a]	6.86 ± 0.64[a]
0.1	6.35 ± 0.59[a]	7.78 ± 1.49[a]	5.63 ± 0.57[a]	8.07 ± 1.10[a]
0.3	8.43 ± 1.18[a]	9.65 ± 2.14[a]	7.11 ± 0.83[a]	11.16 ± 1.21[a]
0.5	95.33 ± 1.02[a]	91.93 ± 1.26[a]	95.64 ± 3.27[a]	89.38 ± 4.74[a]
0.7	99.37 ± 0.45[a]	96.98 ± 1.32[a]	95.90 ± 3.29[a]	95.21 ± 4.20[a]
0.9	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]

[a, b] = Values with the same superscript letters are not significant (P > 0.05).

APPENDIX XIII

Effects of sub-chronic lead exposure and pretreatment with ascorbic acid on thyroid function and serum biochemistry.

Parameters	**Treatment groups (Mean ± SEM)**			
	Control	**Lead (250 mg/kg)**	**Ascorbic acid (100 mg/kg)**	**Lead + ascorbic acid (250 mg/kg + 100 mg/kg)**
Triiodothyronine (ng/ml)	1.04 ± 0.15^{ab}	0.91 ± 0.09^{b}	1.23 ± 0.08^{a}	1.27 ± 0.09^{a}
Thyronine (µg/ml)	3.84 ± 0.32^{a}	3.60 ± 0.22^{a}	3.39 ± 0.24a	3.68 ± 0.29^{a}
Thyrotropin (µIU/ml)	0.45 ± 0.02^{b}	0.49 ± 0.03^{ab}	0.56 ± 0.04^{a}	0.50 ± 0.04^{ab}
Creatine Kinase (IU/L)	2263.40 ± 430.73^{b}	1408.70 ± 274.18^{c}	2635.10 ± 314.95^{ab}	3333.20 ± 408.71^{a}
Total protein (g/L)	81.00 ± 2.14b[c]	80.50 ± 1.14^{c}	84.80 ± 1.59^{a}	84.20 ± 1.79^{ab}
Albumin (g/L)	40.80 ± 1.31^{a}	39.10 ± 0.99^{a}	39.20 ± 1.24^{a}	39.89 ± 0.87^{a}
Globulins (g/L)	40.67 ± 1.73^{b}	42.11 ± 0.90^{b}	45.89 ± 1.49^{a}	44.33 ± 1.39^{ab}
Aspartate amino transferase (IU/L)	225.70 ± 16.59^{a}	161.50 ± 9.65^{b}	187.90 ± 19.01^{ab}	196.56 ± 7.31^{ab}
Alanine amino transferase (IU/L)	96.00 ± 6.59^{a}	91.10 ± 6.68^{a}	93.00 ± 6.00^{a}	85.00 ± 8.39^{a}
Alkaline phosphatase (IU/L)	189.70 ± 17.62^{a}	193.90 ± 6.73^{a}	196.00 ± 13.01^{a}	208.00 ± 6.28^{a}

Lactate dehydrogenase (IU/L)	1835.70 ± 204.68[a]	1077.70 ± 57.66[b]	1490.00 ± 244.30[ab]	1485.56 ± 56.41[ab]
Malondialdehyde concentration (µmol/mg protein)	9.53 ± 0.73[a]	9.63 ± 0.44[ab]	6.77 ± 0.76[c]	7.66 ± 0.82[bc]

[a, b] = Values with different superscript letters are significantly ($P < 0.05$) different, while those with the same superscript letters are not significant ($P > 0.05$).

APPENDIX XIV

The open-field apparatus

APPENDIX XV

Inclined plane apparatus

APPENDIX XVI

Elicitation of sensorimotor reflex used in determining excitability scores

APPENDIX XVII

Step-down inhibitory avoidance learning task apparatus