EFFECT OF FOOD CHAIN-MEDIATED METAL EXPOSURE ON TISSUE OXIDATIVE ENZYMES AND KIDNEY FUNCTION PARAMETERS OF RATS

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DISSERTATION SUBMITTED TO THE POSTGRADUATE SCHOOL IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE (M.Sc.) DEGREE IN BIOCHEMISTRY, DELTA STATE UNIVERSITY, ABRAKA.

MARCH, 2017

CERTIFICATION

I declare that this Dissertation research was independently carried out by me **NJIDEAKA, Ogochukwu Tracy** in the Department of Biochemistry, Faculty of Science Delta State University Abraka for the award of M.Sc. Degree in Biochemistry and has not been carried out by any one for the award of any Diploma or Degree.

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(Researcher)

APPROVAL PAGE

This is to certify that this research work was carried out by **NJIDEAKA, Ogochukwu Tracy** with matriculation number **PG/12/13/214572** in the Department of Biochemistry, Faculty of Science, Delta State University for the award of Master of Science (M.Sc.) Degree in Biochemistry.

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Prof. S.O Asagba **Date**

(***Supervisor***)

_____ _____

Prof. N.J Tonukari **Date**

(*Head of Department*)

DEDICATION

I dedicate this project work to the Almighty God for His infinite mercy.

ACKNOWLEDGEMENTS

I am most grateful to God Almighty for the grace to complete this project. I am also very grateful for the fatherly role played by my supervisor, Prof. S. O. Asagba in seeing to the completion of this project. God bless you sir.

My profound gratitude also goes to my dear mother, T. O. Udemba, my Lecturers, Mary Ja-ani Bishop, my family and all my course mates for always been there for me when I needed them. God richly bless you all. Amen!

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ABSTRACT

In this study, the effects of food-chain mediated exposure of Cd, As and combination of both metals on weight gain, organ/body weight ratio, tissue oxidative enzymes and plasma urea and creatinine levels in rats were evaluated. Catfishes were obtained and into four groups. Fishes in Group A were reared in fresh water, those in Group B in water contaminated with arsenic (0.4mg/100ml/day), those in Group C lived in water contaminated with cadmium (0.4mg/100ml/day) while fishes in Group D lived in water contaminated with arsenic and cadmium (0.4mg/100ml of each contaminant/day). All the fishes received normal feed for the duration (4 weeks) after which they were killed, oven-dried and used as protein source in the experimental diet. Wistar strain of male albino rats (100±150g) were used for the main experiment. The rats were divided into four (4) groups. Rats in group A (control) received feed composed with non-metal exposed fish, rats in Group B (arsenic) were fed with diet containing arsenic-contaminated fish, rats in Group C (cadmium) were fed with diet containing cadmium-contaminated fish while rats in Group D (arsenic+cadmium) were fed with diet containing arsenic + cadmium contaminated fish. The feeding was done daily for twelve weeks after which the animals were weighed and sacrificed under chloroform anesthesia. The liver, kidney, brain, testis, prostrate and heart were harvested for biochemical assays. Blood samples were also taken, centrifuged and the serum used for assays. The results obtained indicate that cadmium and arsenic when administered separately significantly decreased body weight gain of rats. Both metals and their combination also decreased kidney/body weight ratio of rats. Alterations of the oxidative enzymes was also observed in rats administered both metals and their combinations. Thus the findings of the study indicate that these metals were toxic when administered via the food-chain since they affected the ability of the rats to use the oxidative enzymes for biotransformation.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Heavy metals are known to pose a serious threat to human health (Nordberg et al., 2000). Among the heavy metals, cadmium (Cd) and arsenic (As) are known to be present in water, air, food and even cigarette smoke. They are industrial pollutants from battery, plastic and fertilizer industries (Thijssen et al., 2007). Animal studies have shown that Cd can stimulate formation of reactive oxygen species (ROS) and induce damage to various tissues (Alvarez et al., 2007). Cd is known to enhance lipid peroxidation and DNA damage and can induce abnormal expression of the main antioxidant molecules in cells (Thijssen et al., 2007). It has been listed as a chemical substance that is potentially dangerous on a global level and exposure to it must be minimized (El-Sharaky et al., 2007).

WHO (1992) showed that cadmium poses a threat to humans because it can bio-accumulate in tissues and cause damage. This metal enters surface water from the industrial wastes and is found in soil by leaching of sewage sludge through soil (Joshi and Bose, 2002).

Cadmium-induced damage of the liver is manifested by an increase in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the blood (Kowalczyk et al., 2003; Guilhermino et al., 1998). Other organs affected by cadmium are the brain, testis, prostrate and bone (Te isma, 2000). Following oral exposure, Cd is absorbed and delivered to the liver by

endogenous intestinal protein, metallothionein and from the liver it is rapidly redistributed to other organs with the kidney as the main target organ for Cd toxicity (Asagba, 2009). Public health interest in the toxic effects of environmental Cd was awakened by epidemiological evidence linking industrial Cd waste pollution of marine food sources to the outbreak of *itai itai disease* in Japan, a disease characterized by, among others, severe bone disorders and renal tubular lesions (Tomohito et al., 2010).

Arsenic toxicity, on the other hand, is a global health problem affecting many millions of people. Contamination is caused by arsenic from natural geological sources, leaching into aquifers, contaminating drinking water and may also occur from mining and other industrial processes. It is present as a contaminant in many traditional remedies. Arsenic toxicity inactivates up to 200 enzymes, most notably those involved in cellular energy pathways and DNA replication and repair, and is substituted for phosphate in high energy compounds such as ATP. Unbound arsenic also exerts its toxicity by generating reactive oxygen intermediates during their redox cycling and metabolic activation processes that cause lipid peroxidation and DNA damage (Abernathy et al., 1999**)**.

Humans can become exposed to Cd and AS pollutants through the food chain and the air (Hardej and Trombetta, 2004; Valadez-Vega et al., 2011). Arsenic and Cd exposure occurs from inhalation, absorption through the skin and, primarily, by ingestion of, for example, contaminated drinking water. Arsenic in food occurs as relatively non-toxic organic compounds (arsenobentaine and arsenocholine). Seafood, fish, and algae are the richest organic sources (Buchet et al., 1996***)***. These organic compounds cause raised arsenic levels in blood and arsenic intake is higher from solid foods than from liquids including drinking water (Gonzalez et al., 2007). Organic and inorganic arsenic compounds may enter the plant food chain from agricultural products or from soil irrigated with arsenic contaminated water (Abernathy et al., 1999**)**.

The bioaccumulation of Cd and AS in the trophic food chain is a cause of concern since they can have deleterious effects on human health (Ersoy and Celik, 2009; Jarup and Akesson, 2009). Furthermore, fish and seafood are one of the main links between the heavy metal present in the environment and the human health (Ersoy and Celik, 2009; Jarup and Akesson, 2009).

Fish have the ability to accumulate heavy metal in their tissues by the absorption along the gill surface and gut tract wall to higher levels than the toxic concentration in their

environment (Chevreuil et al., 1995). Fish has been acknowledged as an integral component of a well balanced diet, providing a healthy source of energy, high-quality proteins, vitamins and a wide range of other important nutrients (Pieniak et al., 2010). Moreover, fish is a significant source of omega-3 polyunsaturated fatty acids (PUFAs) whose benefits lowering the risk of coronary heart disease and contributing to normal neurodevelopment in children have been widely recognized (Mozaffarian and Wu, 2011; Swanson et al., 2012).

In contrast to the potential health benefits of dietary fish intake, the chemical pollutants contained in these products have emerged as an issue of concern, particularly for frequent fish consumers (Domingo, 2007; Dórea, 2008; Martorell et al., 2011). In this regard, heavy metals contamination is a worldwide-recognized public health hazard because these pollutants are widespread in the environment, including marine ecosystems, from either natural or anthropogenic sources (Lozano et al., 2010). As a consequence, they can be accumulated by marine organisms through exposure to metals present in water and sediments or in the food chain. Thus, diet comprises the main route of exposure to these elements in the general population (Kim and Lee, 2010) and although fish intake has potential health benefits, the presence of metal contamination in seafood has raised public health concerns (Olmedo et al., 2013).

Many studies have examined the effect of Cd and AS toxicity in rats and other mammals (Mahaffey, et al., 1997; Ratnaike et al., 2003; Asagba, 2010; Hughes, 2002), however, only few have examined their toxicity in rats mediated by a food-chain (Asagba, 2010). This study is thus aimed at examining the effect of food-chain mediated cadmium and arsenic exposure on tissue oxidative enzymes and kidney function parameters of rats.

1.2 Statement of Problem

The negative effect of cadmium and arsenic has been well elucidated by various studies. Their high level of abundance in the environment has made them one of the most risky contaminants exposed to man through food and many other substances. In most of the available studies, the natural route of entry of metals to humans is not taken into account. In such studies the metals are either added directly to food or drinking water of the animals or they are introduced into animal by intramuscular or intraperitoneal injection. Thus the findings from these studies may not be reliable since these metals are expected to be biotransformed in plants or lower animals before they are taken in by man. The problem of this study therefore, is targeted at

exploring the role and effect of cadmium and arsenic intoxication singly and combined through a mimicked food chain in rat models.

1.3 General Aims and Objectives of the study

The aim of this study is to evaluate the effect of food chain mediated metal exposure on tissue oxidative enzymes and kidney function parameters of rats.

1.3.1 Specific Objectives of the Study

The specific objectives of the study are to determine the levels of the following parameters in rats after food-chain mediated exposure to Cd, As and combination of both metals:

i. Weight gain and organ/body weight ratio
ii. Rat tissue xanthine oxidase, monoamine oxidase and aldehyde oxidase activities
iii. Plasma urea and creatinine concentrations

1.4 Significance of Study

The potential effect of chronic and acute exposure to arsenic and cadmium has been well defined in animal and human models. The mechanism through which cadmium and arsenic act to cause deleterious effects has been linked to impaired antioxidant metabolism and oxidative stress may play a role. However, the toxicity of metals via the food chain has not been properly addressed and this underscores the importance of the present study.

1.5 Justification of the Study

The present study will provide scientific evidence on the role of the food-chain in the toxicity of metals such as cadmium and arsenic

1.6 Scope of the Study

Study is an experimental investigation on the comparative effect of cadmium, arsenic and a combination of arsenic and cadmium on tissue (liver, kidney, heart, testes prostrate, and brain) oxidase activities (Xanthine Oxidase, Monoamine oxidase aldehyde oxidase) and plasma urea and creatinine concentrations.

CHAPTER TWO

LITERATURE REVIEW

2.1 Cadmium

2.1.1 Occurrence, Exposure and Toxicity

Cadmium (Cd) is an industrial and environmental pollutant, arising primarily from battery, electroplating, pigment, plastic, fertilizer industries, and cigarette smoke. Cd is dangerous because humans consume both plants and animals that absorb Cd efficiently and concentrate it within their tissues (Stohs and Bagchi 1995). Cd shows various mechanisms of toxicity in particular species under different experimental conditions (Iscan *et al.*, 1994, Žikić *et al*., 1996, Waisberg *et al*., 2003).

Cadmium is released to the biosphere from both natural and anthropogenic sources. It is an element that occurs naturally in the earth's crust and got rank 7 of ASTDR's "Top 20 list" (ASTDR, 1999). Percentage of cadmium in the upper soil has been increasing because it is found in insecticides, fungicides, sludge, and commercial fertilizers which are routinely used in agriculture. Dental alloys, electroplating, motor oil, and exhaust are other sources of Cd pollution. Hence, anthropogenic activities have increased Cd magnification in the environment. 10% of total Cd in the environment is derived from natural sources, whereas remaining 90% is derived form anthropogenic activity (Okada *et al.*, 1997). Volcanic activity contributes about

62% of natural emissions and other natural sources include decaying of vegetation (25%) airborne soil particles (12%) and forest fire (2%). It's noncorrosive and cumulative nature has made it very important due to its applications in electroplating or galvanizing. It is also used as colour pigment for paints, plastics, and as a cathode material for nickel-cadmium batteries. Anthropogenic activities like; smelting operations, use of phosphate fertilizers, pigment, cigarettes smokes, automobiles etc. have contributed to the entry of cadmium into human and animal food chain (WHO, 1992; Okada *et al.*, 1997; Kumar *et al.*, 2007). Presence of cadmium at higher concentration than the maximum allowable limits in water, vegetation and food have been reported by various authors (Agarwal and Raj, 1978; Khandekar *et al.*, 1980; Allen, 1995; Laxi, 2005; Kumar *et al.*, 2008; Asagba, 2010).

Higher level of Cd has also been detected in sewage sludge (rich in almost all nutrients and hence generally used as plant fertilizer), various vegetables (Roblenbeck *et al.*, 1999), animals feed and their tissues (Kumar *et al.*, 2007). Topsoil enrich in sludge contributes Cd accumulation in the blood, milk, hair, liver and kidney of sheep, goat, cow, buffalo (Brebner *et al.*, 1993; Swarup *et al.*, 2005; Balagangatharathilagar *et al.*, 2006 and Patra *et al.*, 2007).

It has been demonstrated that Cd stimulates free radical production, resulting in oxidative deterioration of lipids, proteins and DNA, and initiating various pathological conditions in humans and animals (Waisberg *et al*. 2003). Once absorbed, Cd is rapidly cleared from the blood and concentrates in various tissues. Chronic exposure to inorganic Cd results in accumulation of the metal mainly in the liver and kidneys, as well as in other tissues and organs causing many metabolic and histological changes, membrane damage, altered gene expression and apoptosis (Shaikh *et al*. 1999, Casalino *et al*. 2002, Waisberg *et al*. 2003).

Cadmium is known to cause reproductive disorders, renal and hepatic dysfunction, osteomalacia, neurological impairment, pancreatic activity changes (Hooser, 2007). It also affects various structures and metabolic processes, such as nucleic acids, carbohydrates energy metabolism, protein synthesis and enzyme systems (Cinar, 2003). Chronic cadmium toxicity also causes an oxidative stress through lipid peroxidation and consumption of some antioxidant systems (Cinar et al., 2010).

Humans become exposed to Cd pollutants through the food chain and the air. Following oral exposure, Cd is absorbed and delivered to the liver by endogenous intestinal protein metallothionein and from the liver it is rapidly redistributed to other organs with the kidney as

the main target organ for Cd toxicity (Asagba, 2009). Studies with experimental animals have shown that exposure to Cd results in toxic lesions in many species, with Cd showing various mechanisms of toxicity in particular species under different experimental conditions (Waisberg et al., 2003). Severity of Cd intoxication of target organs is dependent on the route, dose, and duration of exposure and it manifests in various forms ranging from acute toxicosis to cancer. Cadmium causes toxic lesions in target organs by inducing oxidative stress (Patra et al., 2011).

2.1.2. Cadmium in the Food Chain

Reports indicate that environmental heavy metals like Cd are mobilized in the food chain affecting producers and consumers (Veltman et al., 2008). To assess the importance of environmental parameters on metal bioavailability, researchers have developed indexes like bioaccumulation factors, defined as the concentration of a chemical in the organism divided by the concentration in the soil. However, the bioconcentration factors are affected by several environmental conditions inherent to the environment and the organisms (Veltman et al., 2008). In an experiment including Cd fed plants at 0–100_gg−1, the primary consumer (the snail *Helix aspersa*) and the secondary consumer (larvae of the beetle *Chrysocarabus splendens*) showed Cd bioaccumulation factors of 1.87–3.9 in *H. aspersa* and less than 1_gg−1 in *C. splendens*, the secondary predator (Scheifler et al., 2002). The study also showed that the exposure to Cd laden snails produced 31% beetle larvae mortality, which demonstrated the potential toxicity of the Cd movement in the food chain. Scheifler et al. (2006) have also reported that snails absorbed the most Cd from soil, not from plants. Recent studies have shown that Cd is transferred from plants to snails in a concentration dependent manner (Gimbert et al., 2008), which corroborate the potential hazard of Cd hyperaccumulator plants.

Another aspect to consider is the effect of Cd on plant predator metabolisms. For instance, in *Neochetina eichhorniae*, the water hyacinth weevils, Cd reduced about 70–80% of protein and 8–20% of lipids in the ovaries of insects fed with leaves of plants treated for a week with 100mgCdkg−1 (Jamil and Hussain, 1993). However, according to Veltman et al. (2008) the assimilation of Cd from food is low.

Food is an important pathway for several metals, particularly in populations consuming regionally contaminated foods. According to Chary et al. (2008), populations who restrict their diet to locally grown produce, such as subsistence farmers, are particularly at risk from soil

contamination, because the Cd in their diet is not diluted by food from other non-contaminated areas, as it is in the majority of the developed world.

Chien et al. (2002) calculate the exposure risk to toxic elements through food by using a quotient called the target hazard quotient (THQ). The THQ includes the exposure frequency and the concentration of the contaminant, among others. They considered that when THQ is lower than 1, the level of daily exposure to the human population was safe. Using the THQ index Zheng et al. (2007a) concluded that inhabitants living 500–1000m from the Huludao Zinc Plant in China have acquired THQ values from vegetables higher than 1, which means they are at risk of Cd toxicity. In addition, Yang et al. (2006) reported that in the upper Wu Jiang River basin, province of Guangdong, China, rice plants irrigated with untreated mining wastewater had 0.24_gCdg−1 in unpolished rice, and the dietary uptake of Cd was calculated to be "2.2 and 1.5_gCdkg−1 bodyweight per day for a 60-kg adult and 40-kg child, respectively."

These values exceed the provisional tolerable dietary intake set by FAO/WHO, which is 1_gCdkg−1 bodyweight. Although, rice is consumed as the staple food in Guangdong, and hence, rice contributes a major part to the total daily food intake, there are other sources of Cd intake, such as dairy products and vegetables that must be considered as part of the risk factors.

According to Millis et al. (2004), the consumption of vegetables is the main source of Cd for humans. These researchers have pointed out that the heterogeneity of soil results in variation in element concentration in plants, which might result in an inaccurate health risk assessment. Another factor that must be considered is the genetic constitution of crops. Antonious and Kochlar (2009) tested the heavy metal uptake capacity of 23 capsicum accessions from the USDA germplasm collection at the Kentucky State University Research Farm, Franklin County, KY. These researchers found that the accession PI-246331 accumulated significantly more Cd than the others.

In several places around the world (e.g. Mexico, Pakistan, and China), cropland is irrigated with wastewater, and studies on metal transfer from crops to humans are just starting to be performed in these countries. Among crop plants, rice has a special place due to its capacity to absorb Cd. Chaney et al. (2004) have reported that rice has the "ability to accumulate soil Cd in grains, excluding Fe, Zn and Ca (even though the soil contains 100 times more Zn than Cd)." This poses a real threat for farmers consuming polished rice that is deficient in Fe, Zn, and Ca (Chaney et al., 2004). According to Khan et al. (2008), the daily intake of Cd from crop plants

normally cultivated in wastewater irrigated land seems to be too low, based on the health risk indexes, to pose a threat for the human population. However, in a study that included data collected for 16 years, Kobayashi et al. (2002) found that in the Jinzu River basin, the increased total Cd intake by humans appears to be related to an adverse effect of this element on life prognosis. These researchers found that the mortality rate in people ingesting more than 2.0mg Cd from rice cultivated in a Cd-polluted area was higher compared to people ingesting less than 2.0mg of Cd.

2.1.3. Cadmium Compounds in Animals

In mammals Cd is transported as a Cd–protein complex, especially Cd–metallothionein, and stored in liver, kidney and intestinal mucosa (Cooke and Johnson, 1996) with a retention time of 10 years (Pokorny and Ribaric-Lasnik, 2000). Cooke and Johnson (1996) have reported that in an ecosystem around a refinery site, the common shrew (*Sorex araneus*), an insectivorous mammal, concentrates 273±15mg Cd kg−1 dry weight in kidneys while the field vole (*Microtus agrestris*), a herbivore mammal, concentrated 88.8±23.3mgCd kg−1 DW in kidneys also. As pointed out by Kan and Meijer (2007), the concentration of Cd in the feed, the duration of exposure to the feed and the chemical form of Cd in the feed can determine the Cd content in liver and kidneys. Although there is abundant information about the toxicity of cadmium and its movement in the food chain, more information is required to elucidate the metabolic pathways followed by Cd compounds obtained by higher consumers of the food chain.

2.1.4 Mechanism of Toxicity

It has been demonstrated that Cd induces oxidative stress by stimulating the production of free radicals such as hydroxyl radicals (Patra et al., 2011), superoxide anions, nitric oxide and hydrogen peroxide (Waisberg et al., 2003), resulting in membrane lipid peroxidation and oxidative deterioration of proteins and DNA and in the process initiating various pathological conditions in humans and animals (Waisberg et al., 2003). Thus, a number of antioxidants and antioxidant defence systems have been shown to protect cells of target organs from Cd toxicity (Swaran, 2009) or to reverse Cd toxicity (Tandon et al., 2003).

The liver and the kidney are two critical organs with respect to Cd toxicity (Nordberg et al., 2000). Cd accumulates mainly in the liver and kidneys, where it causes metabolic changes and abnormal gene expression (Koyu et al, 2006). Elaborate morphological changes have been observed in both organs in cases of Cd poisoning. The mechanism of Cd-induced hepatotoxicity and renal toxicity is poorly understood.

2.2 Arsenic

2.2.1 Occurrence and Uses

Arsenic is one of the most toxic metals derived from the natural environment. The major cause of human arsenic toxicity is from contamination of drinking water from natural geological sources rather than from mining, smelting, or agricultural sources (pesticides or fertilisers) (Matschullat, 2000). Prolonged ingestion of water contaminated with arsenic may result in the manifestations of toxicity in practically all systems of the body. The most serious concern is the potential of arsenic to act as a carcinogen.

Over the centuries, arsenic has been used for a variety of purposes. Arsenic was a constituent in cosmetics, and used more extensively than at present in agriculture to protect crops from pests. Arsenic as copper acetoarsenite was a pigment in paints, the best known being “Paris green”. Before electricity was used for illumination, hydrogen liberated from coal fires and from gas for lighting combined with arsenic in the Paris green used in wallpaper to form arsine, a toxic gas. A fungus *Scopulariopsis breviculis* present in damp wallpaper also metabolised the arsenic in Paris green to arsine (Matschullat, 2000).

In industry, arsenic is used to manufacture paints, fungicides, insecticides, pesticides, herbicides, wood preservatives, and cotton desiccants. As it is an essential trace element for some animals, arsenic is an additive in animal feed. Gallium arsenide or aluminium gallium

arsenide crystals are components of semiconductors, light emitting diodes, lasers, and a variety of transistors.

Arsenic is a popular murder weapon. Many arsenic compounds resemble white sugar and this apparent innocuousness is enhanced by being tasteless and odourless and was publicised by Frank Capra's film *Arsenic and Old Lace*, in which two elderly ladies use arsenic in elderberry wine to murder their male suitors (Matschullat, 2000).

2.2.2 Chemistry and Toxicity

Arsenic occurs in two oxidation states: a trivalent form, arsenite (As_2O_3; As III) and a pentavalent form, arsenate (As_2O_5; As V). As III is 60 times more toxic than As V. Organic arsenic is non-toxic whereas inorganic arsenic is toxic. Arsenic toxicity inactivates up to 200 enzymes, most notably those involved in cellular energy pathways and DNA replication and repair, and is substituted for phosphate in high energy compounds such as ATP. Unbound arsenic also exerts its toxicity by generating reactive oxygen intermediates during their redox cycling and metabolic activation processes that cause lipid peroxidation and DNA damage (Abernathy et al., 1999**)**. As III, especially, binds thiol or sulfhydryl groups in tissue proteins of the liver, lungs, kidney, spleen, gastrointestinal mucosa, and keratin-rich tissues (skin, hair, and nails).

Klaassen et al., (2003) reported that Arsenic and many of its compounds are especially potent poisons. Arsenic disrupts ATP production through several mechanisms. At the level of the citric acid cycle, arsenic inhibits pyruvate dehydrogenase and by competing with phosphate it uncouples oxidative phosphorylation, thus inhibiting energy-linked reduction of NAD+, mitochondrial respiration, and ATP synthesis. Hydrogen peroxide production is also increased, which might form reactive oxygen species and oxidative stress. These metabolic interferences lead to death from multi-system organ failure probably from necrotic cell death, not apoptosis.

Arsenite inhibits not only the formation of acetyl-CoA but also the enzyme succinic dehydrogenase. Arsenate can replace phosphate in many reactions. It is able to form Glc-6-Arsenate in vitro; therefore it has been argued that hexokinase could be inhibited (Hughes, 2000). (Eventually this may be a mechanism leading to muscle weakness in chronic arsenic poisoning.) In the glyceraldehyde-3-P-dehydrogenase reaction arsenate attacks the enzyme-

bound thioester. The formed 1-arseno-3-phosphoglycerate is unstable and hydrolyzes spontaneously. Thus, ATP formation in Glycolysis is inhibited while bypassing the phosphoglycerate kinase reaction. (Moreover, the formation of 2,3-bisphosphoglycerate in erythrocytes might be affected, followed by a higher oxygen affinity of hemoglobin and subsequently enhanced cyanosis). As shown by Gresser (1981), submitochondrial particles synthesize Adenosine-5'-diphosphate-arsenate from ADP and arsenate in presence of succinate. Thus, by a variety of mechanisms arsenate leads to an impairment of cell respiration and subsequently diminished ATP formation (Gresser, 1981). This is consistent with observed ATP depletion of exposed cells and histopathological findings of mitochondrial and cell swelling, glycogen depletion in liver cells and fatty change in liver, heart and kidney.

2.2.3 Arsenic Exposure

Arsenic exposure occurs from inhalation, absorption through the skin and, primarily, by ingestion of, for example, contaminated drinking water. Arsenic in food occurs as relatively non-toxic organic compounds (arsenobentaine and arsenocholine). Seafood, fish, and algae are the richest organic sources (Buchet et al., 1996). These organic compounds cause raised arsenic levels in blood but are rapidly excreted unchanged in urine. Arsenic intake is higher from solid foods than from liquids including drinking water. Organic and inorganic arsenic compounds may enter the plant food chain from agricultural products or from soil irrigated with arsenic contaminated water (Thomas *et al*., 1999).

2.3.3.1 Arsenic in the Environment

Arsenic is a metalloid of great environmental concern due to its toxicity and abundance. In countries such as Bangladesh, China, Hungary, and India, among others, As is found at high concentration in ground water and surface soil (Chen et al., 2006). Arsenic disperses into soil and water through the disintegration of rocks and minerals and lixiviation (the process of removing soluble constituents of matter by liquid permeation) (Luong et al., 2007). Beside its natural incidence, As is also released into the environment from smelting and mining processes, agricultural practices, fabrication and consumption of wood preservatives, and food additives (Aldrich et al., 2007).

The mobility and availability of As in the environment depends on its chemical form and speciation. Inorganic arsenic in the form of arsenite (As(III)) is less mobile but has been

considered more toxic than inorganic arsenate (As(V)). However both species have been considered harmful to living organisms due to their capability to alter metabolic pathways (Caruso et al., 2001). Arsenic (III) binds to sulfur groups from enzymes and proteins and to thiol groups from phytochelatins, whereas As(V) binds to amino, or reduced nitrogen groups (Kumaresan and Riyazuddin, 2001). Detoxification of As occurs through methylation of inorganic As(III) which is highly metabolized by humans. This results in the production of organic arsenic compounds such as monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), and trimethylarsine which are also considered to be cancer promoters (Zhou et al., 2003). According to Heikens (2006), As(V) is the most common and stable form of As found in aerobic soils and is therefore more available for plant uptake. The transport and availability of As in soil is strongly dependent on the soil pH. At low pH values (pH 4) As is found complexed with iron whereas at high pH values (pH 6–8) it is mostly bound to calcium (Fayiga et al., 2007). Moreover, the presence of Fe and manganese oxides also increases As mobility and availability in soil (Zavala and Duxbury, 2008)

2.2.4 Arsenic in the Food Chain

Animals can absorb As from different sources but do so mainly from the soil matrix. A study performed by Gongalsky et al. (2004) has shown that the beetle *Blaps rugosa* living in tailing ponds from a metallurgic plant contained 29mgAs kg−1, while *Poecilus gebleri, P. fortipes*, and *Nicrophorus investigator* contained As in a range of 0.6–3.0mgkg−1. However, the authors do not study the form or complexity of As in the animals. Conversely, Morrissey et al. (2007) found that in bark beetles As was found as MMA and DMA.

This might suggest that the beetles do not metabolize the MMA compound. Milton and Johnson (1999) reported on As transport from plants to invertebrate herbivores, in an ecosystem formed at a tailing pond containing As at 630±34mgkg−1. Plants collected from the tailing (*Trifolium repens*, *Festuca rubra*, and *Agrostis stolonifera*) were shown to have As concentrations varying from 0.5 to 1.1mgAskg−1 DW. Gastropods such as slugs (*Arion ater*) living on the tailings surface had higher As concentrations (10mgAs kg−1 DW) in their tissues than those living in pond wall (3mgAs kg−1 DW). In contrast, As concentrations in tissues from snails (*Cepaea nemoralis*)was higher (5mgAs kg−1 DW) insnails living on the pond walls that those living on the tailings surface (3mgAs kg−1 DW).

Both species feed on vegetation, fungi, algae, and lichens; however, *A. ater* feeds also on animal debris. These studies show the As transfer from soil-living organisms to the higher trophic level predating snails and slugs.

Humans can take up As in several ways including inhalation, smoking, water or food (Jaerup and Pershagen, 1991). Due to the toxicity of As, the World Health Organization (WHO, 1993) has set a value of 2_gAskg−1 bodyweight/day as the tolerable daily intake.

However, in countries where most of the basic diet consists of vegetables, the stated value is not observed when cropland or irrigation water contain high levels of As (Sengupta et al., 2006; Signes-Pastor et al., 2008).Worldwide, rice is one of the basic crops known for its high potential to accumulate As. This is because it is often watered with groundwater containing elevated As concentrations (Zhu et al., 2008). Recently, Ma and researchers reported that also As(III) is transported in the form of arsenous acid (As(OH)3) through silicic acid transporters (Lsi1 and Lsi2) in the rice cultivars Oochikara, T-65, and Koshihikari (Ma et al., 2008).

In some countries, the As levels in rice are 10-fold higher when compared to other cereal crops (Zavala and Duxbury, 2008). The situation is worse in countries where food is cooked with water containing As (Roychowdhury, 2008). Arsenic can be found in some rice grains as inorganic As in a range between 10 and 90% and the remaining percentage as organic As. However, it also depends on several factors such as the As concentration in soils, As bioavailability, rice cultivars, and country of origin, among others. Meharg et al. (2008) reported a difference between the total As found in brown rice obtained from paddy fields from countries like Bangladesh, China, and US (0.61, 0.36, and 0.44mgAs kg−1, respectively). These researchers also reported that white rice contained 0.28mgAs kg−1 and brown rice contained 0.44mgAskg−1 DW. Yet, inorganic As represents the highest risk for human dietary intake since it can bind to important molecule such as proteins or DNA (Zhu et al., 2008). Rahman et al. (2008) reported that rice cultivated in soils containing 60mgAs kg −1 had about 27.3±0.44mgkg−1 in the straw and 1.6±0.52mgkg−1 DW in the husk. Use of straw and husk as feedstocks for cattle could be a route for As accumulation in human body. Trophic transfer of As occurs mainly in countries like India and Bangladesh where the health effects of As in people are well documented (Devesa et al., 2001; Del Razo et al., 2002).

2.2.5 Arsenic Complexes in Animals

Besides As intake from cereals and vegetables, consumption of certain seafood can be a potential source of As intake. The As concentration in marine animals can be double or triple compared with As in terrestrial foods (Schoof et al., 1999). According to Rose et al. (2007), As is first accumulated in phytoplankton from sea water and transformed into arsenosugar compounds and DMA, which are taken up by algae. Arsenosugar compounds are then transformed to arsobetaine by methylation of AsV, MA, and DMA by microbial organisms. The As levels can be magnified 1000–10,000-fold in marine animals that ingest algae (Borak and Hosgood, 2007).

These researchers suggested that arsenocholine, which was found in very small amounts, seems to be a metabolic intermediate of arsenobetaine. Arsenobetaine was mainly found in muscle, while simple inorganic As compounds and DMA were found in viscera of marine animals. In a study on relating to Chinese food Li et al. (2003) revealed the biotransformation of arsenosugar-PO4 to DMA after algae ingestion by shrimp. Furthermore, bivalves such as oysters, mussels and clamswere found to accumulate arsenosugar-PO4 and arsenobetaine, which are less toxic than inorganic As compounds.

The dietary intake of As via seafood depends on the type of animal tissue consumed by humans (Borak and Hosgood, 2007). Cooking processes such as boiling, baking, and stewing, among others, can increase organic and inorganic As in bivalves and squids (Del Razo et al., 2002; Devesa et al., 2001). Mandal et al. (2007) reported the presence of As(III), As(V), arsenobetaine, MMA, and DMAin urine while only arsenobetaine, MMA, and DMA were found in red blood cells of blood plasma from human populations living in the As-contaminated areas of West Bengal, India. These researchers conclude that daily diet including crops grown in As-contaminated sites, is the main source of As transfer to humans. However, a balanced diet rich in protein can help to amend the adverse effects of As on humans by increasing the As metabolism (Singh et al., 2008).

2.2.6 Absorption and Metabolism

The major site of absorption is the small intestine by an electrogenic process involving a proton (H^+) gradient. The optimal pH for arsenic absorption is 5.0, though in the milieu of the small bowel the pH is approximately 7.0 due to pancreatic bicarbonate secretion (Gonzalez, 1997).

The absorbed arsenic undergoes hepatic biomethylation to form monomethylarsonic acid and dimethylarsinic acid that are less toxic but not completely innocuous (Aposhian, 1997).

About 50% of the ingested dose may be eliminated in the urine in three to five days. Dimethylarsinic acid is the dominant urinary metabolite (60%–70%) compared with monomethylarsonic acid (Benramdane et al., 1999). A small amount of inorganic arsenic is also excreted unchanged. After acute poisoning electrothermal atomic absorption spectrometry studies show that the highest concentration of arsenic is in the kidneys and liver (Benramdane et al., 1999).

In chronic arsenic ingestion, arsenic accumulates in the liver, kidneys, heart, and lungs and smaller amounts in the muscles, nervous system, gastrointestinal tract, and spleen. Though most arsenic is cleared from these sites, residual amounts remain in the keratin-rich tissues, nails, hair, and skin. After about two weeks of ingestion, arsenic is deposited in the hair and nails (Benramdane et al., 1999).

2.2.7 Acute and Chronic Poisoning

Most cases of acute arsenic poisoning occur from accidental ingestion of insecticides or pesticides and less commonly from attempted suicide. Small amounts (<5 mg) result in vomiting and diarrhoea but resolve in 12 hours and treatment is reported not to be necessary. The lethal dose of arsenic in acute poisoning ranges from 100 mg to 300 mg (Kingston et al., 1993).

The clinical features initially invariably relate to the gastrointestinal system and are nausea, vomiting, colicky abdominal pain, and profuse watery diarrhoea. The abdominal pain may be severe and mimic an acute abdomen. Excessive salivation occurs and may be the presenting complaint in the absence of other gastrointestinal symptoms (Ghariani et al., 2001). Other clinical features are acute psychosis, a diffuse skin rash, toxic cardiomyopathy, and seizures (Greenberg et al., 1999).

Haematological abnormalities reported are haemaglobinuria, intravascular coagulation, bone marrow depression, severe pancytopenia, and normocytic normochromic anaemia and basophilic stippling. Renal failure was reported in four of eight sailors exposed to arsine. Respiratory failure and pulmonary oedema are common features of acute poisoning (Lerman et al., 1990).

Long term arsenic toxicity leads to multisystem disease and the most serious consequence is malignancy. The clinical features of arsenic toxicity vary between individuals, population groups, and geographic areas. It is unclear what factors determine the occurrence of a particular clinical manifestation or which body system is targeted. Thus in persons exposed to chronic arsenic poisoning, a wide range of clinical features are common. The onset is insidious with non-specific symptoms of abdominal pain, diarrhoea, and sore throat (Lerman et al., 1990).

2.3 Accumulation of Metals by Fish Living In Polluted Waters

Fish living in polluted waters tend to accumulate heavy metals in their tissues. Generally, accumulation depends on metal concentration, time of exposure, way of metal uptake, environmental conditions (water temperature, pH, hardness, salinity), and intrinsic factors (fish age, feeding habits). Various metals show different affinity to fish tissues. Most of them accumulate mainly in liver, kidney and gills. Fish muscles, comparing to the other tissues, usually contain the lowest levels of metals (Barbara, et al., 2006).

Generally, the higher metal concentration in the environment, the more may be taken up and accumulated by fish. Relationship between metal concentrations in fish and in the water was observed in both, field and laboratory studies (Moiseenko et al., 1995; Linde et al., 1996; Yamazaki et al., 1996; Zhou et al., 1998). It should be, however, emphasized that body metal level is related to its waterborne concentration only if metal is taken up by the fish from water. In most cases, fish from metal-contaminated water are safe for human consumption due to low metal accumulation (except for mercury) in the muscle tissue. However, such fish may constitute a potential risk for predatory fishes, birds and mammals feeding on contaminated fish.

The cadmium-related contamination of the aquatic habitat has greatly increased in the last decades, resulting in an increase of cadmium deposits in tissues of aquatic organisms in all food chain systems (Giles, 1988). It is important to note that cadmium is a highly toxic element for all mammals and fish. Cadmium levels have constantly been increasing, and consequently, the research on cadmium has become quite topical and urgent. Accumulation of cadmium in living organisms is a major ecological concern, especially because of its ability to accumulate very quickly. By contrast, the excretion of cadmium from living organisms is a slow process. In fish, cadmium can cause a number of structural and pathomorphological changes in various organs. The highest cadmium levels were detected in the kidneys and liver of fish (Thophon *et al*.,

2003). Cadmium is noted for its tendency to accumulate in the organisms of mammals for a prolonged biological semi- life. It is responsible for increased hypertension, emphysema, kidney tubule damage, impaired liver function, and cancer (Ribelin and Migaki, 1975).

2.3.1 Site for Absorption in Fish and Molecular Mechanisms of Absorption

In the fish, the possible areas of absorption of dissolved metals are the gills (respiratory tract), the intestine (ingestive intake) and the skin (transcutaneous uptake).

There are various mode of Cd uptake in aquatic organism, where it is most readily absorbed by organisms directly from the water in its free ionic form Cd (II) (AMAP 1998). Metal ions are usually absorbed through passive diffusion or carrier mediated transport over the gills while metals associated with organic materials are ingested and absorbed by endocytosis through intestine. It has been suggested that cadmium ions enter the chloride cells in the gills through calcium channels (Olsson,, 1998). Once enter in the cells the metal is made available for the interaction with cytoplasmatic components such as enzymes (causing toxic effects) and Metallothioneine (probably being detoxified). Although Metallothoneine is induced in the gills it does not appear to be as capable of sequestering the vast majority of accumulated Cd2+, as it is in the liver (Olsson and Hogstrand, 1987). The reason for this is believed to be due to the high affinity of Cd2+ for Ca2+ binding sites in the gills (Flick *et al.*, 1987), and it is also believed that Cd2+ binds to the active sites on the basolateral Ca2+-pump in chloride cells. It thus seems that Cd2+ enters the gills through Ca-channel on the apical side and is further translocated to the circulation interactions with Ca2+-ATPases on the basolateral side.

2.3.2 Tissue Distribution:

Bioaccumulation of cadmium takes place at tropic level and found to be highest in algae (Ferard *et al.*,1983; Pinto *et al.*, 2003). It also accumulates in considerable concentrations in various organs of fish (Sindayigaya *et al.*, 1994; Kumar *et al.*, 2006; Kumar *et al.*, 2008). Smet & Blust (2001) reported that cadmium accumulates in tissues of carp Cyprinus carpio in following order: kidney> Liver> Gills. Kumar *et al.* (2005) have also reported similar accumulation pattern in *Clarias batrachus* in an experimental study. Some insects can also accumulate high levels of cadmium without showing any adverse effects (Jamil and Hussain, 1992). Kidney is the prime target organ for cadmium. The liver also stores a considerable part of the accumulated cadmium. Cadmium is redistributed to these organs directly following uptake through the gills and

intestine, but there may also be redistribution of cadmium form other organs (Olsson and Hogstrand, 1987).

2.4 Tissue Oxidative Enzymes

2.4.1 Sulfite Oxidase

Sulfite oxidase (EC 1.8.3.1) is an enzyme in the mitochoadria of all eukaryotes. It oxidizes sulfite to sulfate and, via cytochrome c, transfers the electrons produced to the electron transport chain, allowing generation of ATP in oxidative phosphorylation (DTh-rico, et al., 2006; Tan, et al., 2005). This is the last step in the metabolism of sulfur-containing compounds and the sulfate is excreted.

Suffite oxidase is a metallo-enzyme that utilizes a molybdopterin cofactor and a heme group. It is one of the cytochromes b5 and belongs to the enzyme super-family of molybdenum oxotransferases that also includes DMSO reductase, xanthine oxidase, and nitrite reductase. In mammals, the expression levels of sulfite oxidase is high in the liver, kidney, and heart, and very low in spleen, brain, skeletal muscle, and blood.

2.4.1.1 Structure

As a homodimer, sulfite oxidase contains two identical subunits with an N-terminal domain and a C- terminal domain. These two domains are connected by ten amino acids forming a ioop. The N-terminal domain has a heme cofactor with three adjacent antiparallel beta sheets and five alpha helices. The C- terminal domain hosts a molybdopterin cofactor that is surrounded by thirteen beta sheets and three alpha helices. The molybdopterin cofactor has a Mo(VI) center, which is bonded to a sulfur from cysteine, an enedithiolate from pyranopterin, and two terminal oxygens. It is at this molybdenum center that the catalytic oxidation of sulfite takes place.

2.4.1.2 Deficiency

Sulfite oxidase is required to metabolize the sulfur-containing amino acids cysteine and methionine in foods. Lack of functional sulfite oxidase causes a disease known as sulfite oxidase deficiency. This rare but fatal disease causes neurological disorders, mental retardation, physical deformities, the degradation of the brain, and death. Reasons for the lack of functional sulfite oxidase. Include a genetic defect that leads to the absence of a molybdopterin cofactor and point mutations in the enzyme (Karakas,. 2005).

2.4.2 Aldehyde Oxidase

Aldehyde oxidase (AO) is a metabolizing enzyme, located in the cytosolic compartment of tissues in many organisms. AO catalyzes the oxidation of aldehydes into carboxylic acid, and in addition, catalyzes the hydrozylation of some heterocycles (Gordon et al, 1940). It can also catalyze the oxidation of both cytochrome P450 (CYP45O) and monoamine oxidase (MAO) intermediate products. AO plays a very important role in the metabolization of numerous drugs.

2.4.2.1 Reaction

AO catalyzes the conversion of an aldehyde in the presence of oxygen and water to an acid and hydrogen peroxide.

- an aldehyde $+H_20+0_2$ <=> a carboxylate + H_20_2 + H^+

Though the enzyme uses molecular oxygen as an electron acceptor, the oxygen atom that is incorporated into the carboxylate product is from water; however, the exact mechanism of reduction is still not known for A0.

The A0 also catalyzes the oxidation of heterocycles, which involves a nucleophilic attack located at the carbon atom beside the heteroatom. This means that susceptibility to nucleophilic attack of a heterocycle determines if that heterocycle is a suitable substrate for A0.

2.4.4.2 Species Distribution

Aldehyde oxidase is a member of the molybd-flavo protein family (Gordon etal., 1940) and has a very complex evolutionary profile—as the genes of AO varies according to animal species (Garattini et al., 2012). Higher primates, such as humans, have a single functioning A0 gene (AOX1), whereas rodents have four separate AOX genes. The human population has both functionally inactive hAOX1 allelic variants and encoding enzyme variants with different catalytic activities. A0 activity has been found to be much more active in higher primates (compared to rodents), though many factors may affect this activity, such as gender, age, cigarette smoking, drug usage, and disease states.

2.4.2.3 Tissue Distribution

Aldehyde oxidase is very concentrated in the liver, where it oxidizes multiple aldehydes and nitrogenous heterocyclic compounds, such as anti-cancer and immunosuppressive drugs (Gordon etal., 1940). Some A0 activity has been located in other parts of the body--including the lungs (epithelial cells and alveolar cells), the kidneys, and the gastrointestinal tract (small and large intestines).

2.4.2.4 Role in Drug Metabolism

Aldehyde oxidase is thought to have a significant impact on pharmacokinetics. A0 is capable of oxidizing many drugs in the liver (such as N-1-methylnicotinamide, N-methylphthalazinium, benzaldehyde, retinal, and vanilhin), because of its broad substrate specificity (Strelevitz, 2012). A0 greatly contributes to the hepatic clearance of drugs and other compounds (Ilartmann, 2012). For example, cytoplasmic AOX1 a key enzyme in the hepatic phase Imetabolism of several xenobiotics (Garattim *et al*., 2012). For this reason, AOX genes are becoming increasingly important to both understand and control in the therapeutic drug industry (Garattini Ct al., 2012).

2.4.3 Monoamine Oxidase

L-Monoamine oxidases (MAO) (EC 1.4.3.4) are a family of enzymes that catalyze the oxidation of monoammes (Tipton, et al., 2004; Edmondson, et al., 2004). They are found bound to the outer membrane of mitochondria in most cell types in the body. The enzyme was originally discovered by Mary Bemheim in the liver and was named tyramine oxidase (Hare, 1928; Slotkin, 1999). They belong to the protein family of flavin-containing amine oxidoreductases.

2.4.3.1 Subtypes and Tissue Distribution

In humans there are two types of MAO: MAO-A and MAO-B (Shih, et al., 2004)

• Both are found in neurons and astroglia.

• Outside the central nervous system:

- MAO-A is also found in the liver, gastrointestinal tract, and placenta. l)
- MAO-B is mostly found in blood platelets.

2.4.3.2 Function

Monoamine oxidases catalyze the oxidative deamination of monoamines. Oxygen is used to remove an amine group from a molecule, resulting in the corresponding aldehyde and ammonia. Monoamine oxidases contain the covalently bound cofactor FAD and are, thus, classified as flavoproteins.

2.4.3.3 Substrate Specificities

They are well known enzymes in pharmacology, since they are the substrate for the action of a number of monoamine oxidase inhibitor drugs. MAO-A is particularly important in the catabolism of monoamines ingested in food. Both MAOs are also vital to the inactivation of monoaminergic neurotransmitters, for which they display different specificities.

• Serotonin, melatonin, noradrenaline, and adrenaline are mainly broken down by MAO-A.

• Phenethylamine and benzylamine are mainly broken down by MAO-B.

• Both forms break down dopamine, tyramine, and tryptamine equally (Kalgutkar, 2001). Specific reactions catalyzed by MAO include:

• Adrenaline or noradrenaline to 3,4-Dihydroxymandelic acid

• Metanephrine or normetanephrine to vanillylmandelic acid (VMA)

• Dopamine to dihydroxyphenylacetic acid

• 3-Methoxytyramine to homovanillic acid

2.4.3.4 Clinical Significance

Because of the vital role that MAOs play in the inactivation of neurotransmitters, MAO dysfunction (too much or too little MAO activity) is thought to be responsible for a number of psychiatric and neurological disorders. For example, unusually high or low levels of MAOs in the body have been associated with depression (Meyer et al, 2006), schizophrenia (Domino et al., 1976), substance abuse, attention deficit disorder, migraines, and irregular sexual maturation. Monoamine oxidase inhibitors are one of the major classes of drug prescribed for the treatment of depression, although they are often last-line treatment due to risk of the drug's interaction with diet or other drugs. Excessive levels of catecholamines (epinephrine, norepinephrine, and dopamine) may lead to a hypertensive crisis, and excessive levels of serotonin may lead to serotonin syndrome.

In fact, MAO-A inhibitors act as antidepressant and antianxiety agents, whereas MAO-B inhibitors are used alone or in combination to treat Alzheimer's and Parkinson's diseases (Riederer, et al., 2011). PET research has shown that MAO is also heavily depleted by use of tobacco cigarettes (Fowler, et al., 1998).

2.4.4 Xanthine Oxidase

Xanthine oxidase (XO, sometimes 'XAO') is a form of xanthine oxidoreductase, an enzyme that generates reactive oxygen species (Ardan, et al., 2004). It catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid. This enzyme plays an important role in the catabolism of purines in some species, including humans (Rule, 2005; Harrison, 2002).

Xantbine oxidase is defined as an enzyme activity (EC 1.17.3.2) The same protein, which in humans has the HGNC approved gene symbol XDH, can also have xanthine dehydrogenase activity EC 1.17.1.4). Most of the protein in the liver exists in a fornt with xanthine dehydrogenase activity, but it can be converted to xanthine oxidase by reversible sulfliydryl oxidation or by irreversible proteolytic modification.

2.4.4.1 Reaction

The following chemical reactions are catalyzed by xanthine oxidase:

• hypoxanthine + H20 +02 xantMne ± H202

• xanthine +1120+02 uric acid + 11202

• Xanthine oxidase can also act on certain other purines, pterins, and aldehydes. For example, it efficiently converts 1 -methylxanthine (a metabolite of caffe1ne to 1 -methyluric acid, but has little activity on 3-methyixanthine (Birkett, 1997).

• Under some circumstances it can produce superoxide ion RH +1120 +202 R0H +2 02J +2 H.

2.4.4.2 Catalytic Mechanism

The active site of XO is composed of a molybdopterin unit with the molybdenum atom also coordinated by terminal oxygen (oxo)., sulfur atoms and a terminal hydroxide. In the reaction with xanthine to form uric acid, an oxygen atom is transferred from molybdenum to xanthine, whereby several intermediates are assumed to be involved (Metz, 2009). The reformation of the active molybdenum center occurs by the addition of water. Like other known molybdenum-containing oxidoreductases, the oxygen. atom introduced to the substrate by X0 originates from water rather than from dioxygen (0_2).

2.4.4.3 Clinical Significance

Xanthine oxidase is a superoxide-producing enzyme found normally in serum and the lungs, and its activity is increased during influenza A infection (Hille, 2006).

During severe liver damage, xanthine oxidase is released into the blood, so a blood assay for X0 is a way to determine if liver damage has happened. As well, because xanthine oxidase is

a metabolic pathway for uric acid formation, the xanthine oxidase inhibitor allopurinol is used in the treatment of gout. Since xanthine oxidase is involved in the metabolism of 6-mercaptopurine, caution should be taken before administering allopurinol and 6- mercaptopurine, or its prodrug azathioprine, in conjunction.

Xanthinuria is a rare genetic disorder where the lack of xanthine oxidase leads to high concentration of xanthine in blood and can cause health problems such as renal failure. There is no specific treatment, sufferers are advised by doctors to avoid foods high in purine and to maintain a high fluid intake. Type I xanthinuria has been traced directly to mutations of the XDH gene which mediates xanthine oxidase activity. Type II xanthinuria may result from a failure of the mechanism which inserts sulfur into the active sites of xanthine oxidase and aldehyde oxid.ase, a related enzyme with some overlapping activities (such as conversion of allopurinol to oxypurinol (Dawson, 2006).

Inhibition of xanthine oxidase has been proposed as a mechanism for improving cardiovascular health (Dawson, 2006). Both xanthine oxidase and xanthine oxidopreductase are also present in corneal epithelium and endothelium and may be involved in oxidative eye injury (Cejková, 2002).

2.5 Kidney Function Parameters

Kidney function can be evaluated from the concentrations of plasma or urine analytes, which are mainly dependent on their elimination (e.g., P-Creatinine). These indirect markers can be easily and rapidly measured, but their sensitivity is poor and generally remains unaltered until 75% of renal function has been lost and their concentrations may be modified by extrarenal factors. Direct tests of kidney function are based on the elimination kinetics of markers of glomerular filtration, blood flow, or tubule reabsorption/secretion and are based on the clearance concept. These tests are more difficult and take longer to perform but allow earlier detection of reduced function (Jean-Pierre and Herve, 2008).

Renal or kidney disease is a pathological process affecting any part of the kidney and may or may not be associated with alterations in kidney function. Kidney or renal failure (insufficiency) is characterized by a decrease in one or several kidney functions, first the urine concentrating ability, then the elimination of small-molecular-weight molecules from the plasma, characterizing azotemia (i.e., increases of P-Urea and/or P-Creatinine). Uremia is the syndrome resulting from renal failure. Cases of azotemia are not always primary renal azotemia caused by

parenchymal damage but may be prerenal or postrenal azotemia resulting from reduced kidney perfusion and interferences with urine excretion, respectively (DiBartola, 2005b).

2.5.1 Creatinine

Creatinine is derived from creatine and creatine phosphate in muscle tissue and may be defined as a nitrogenous waste product. Creatinine is not reutilized but is excreted from the body in the urine via the kidney. It is produced and excreted at a constant rate which is proportional to the body muscle mass. As a consequence of the way in which creatinine is excreated by the kidney, creatinine measurement is used almost exclusively in the assessment of the kidney function. Creatinine is regarded as the most useful endogenous marker in diagnosis and treatment of the kidney disease. The plasma level of creatinine is independent of protein ingestion, water intake, rate of urine production and exercise. Elevation of plasma creatinine is an indication of under excretion suggesting kidney impairment since the rate of excretion is constant (DiBartola, 2005b)..

2.5.2 Urea

Urea is also known as carbamide. It is a waste product of many living organism, and is the major organic component of the urine. In humans or terrestrial mammals, the liver convert ammonia to a non-toxic compound, urea, which can be safety transported in blood to the kidney, where it is eliminated in urine. An adult typically excretes about 25grams of urea per day (DiBartola, 2005b).

2.6 Review of Related Works

Asagba (2010) carried out a study on the biochemical changes in urine and plasma of rats in food chain-mediated cadmium toxicity. The food chain was mimicked by exposing rats to diet containing milled Cd-exposed catfish as the source of protein. The toxicity of Cd was monitored by the determination of indices of oxidative stress and kidney dysfunction in plasma and urine of the rats after 1- and 3-month exposure periods. The results obtained indicate that Cd exposure in the rats was followed by marked accumulation of the metal in the liver and kidney with time. However, the kidney accumulated more Cd relative to the liver after both exposure periods. The levels of glucose, creatinine and lipid peroxidation were significantly ($p < 0.05$) increased in both plasma and urine of the rats after 1 month of exposure. Conversely, rats fed the test diet for 3 months had significantly ($p < 0.05$) decreased level of lipid peroxidation in the plasma and urine.

However, the plasma and urine glucose concentrations were restored to levels not significantly ($p > 0.05$) different from control in rats fed the test diet for 3 months. Similarly, after 3 months, the creatinine level in the plasma was also restored to a level not significantly ($p > 0.05$) different from that of the control but was significantly ($p < 0.05$) elevated in the urine. A significant increase in urine protein concentration was demonstrated after 1-month exposure to Cd, but no significant change occurred in the plasma. Rats fed diet containing the Cd-incorporated fish for 3 months also had no significant ($p > 0.05$) change in plasma and urine protein concentrations. He concluded that this route of exposure caused kidney impairment, oxidative stress as well as alteration in glucose metabolism after 1 month but not after 3 months, possibly due to adaptation occasioned by the antioxidative defense armory of the rats.

Mahaffey et al., (2008) studied the effects of concurrent exposure of lead, cadmium, and arsenic on tissue metal accumulation in rats. Male rats were exposed to dietary Pb (200 ppm), Cd (50 ppm), or As (50 ppm) as arsenate either alone or in combination for 10 weeks using a 2 x 2 x 2 factorial design. Cd and As reduced weight gain even when differences in food intake were taken into account, and administration of both Cd and As depressed weight gain more than did either metal alone. Pb did not adversely affect food consumption or weight gain. Increased RBCs were observed after administration of Pb, Cd, or As, and more cells were observed when two or three metals were concomitantly administered. Despite increased numbers of circulating RBCs, hemoglobin and hematocrit were reduced, especially with the Pb-Cd combination. Analysis of blood chemistries showed normal ranges for blood urea nitrogen, creatinine, cholesterol, calcium, albumin, total protein, and bilirubin. Uric acid was increased by Pb, but not by Cd or As. SGOT activity was reduced by As alone. Serum alkaline phosphatase was reduced by either As or Cd but not Pb. Combinations of As and Cd did not further reduce the activity of this enzyme. Kidney weight and kidney weight/body weight ratios were increased by Pb alone, but Cd or As alone or in combination had no effect. Liver weight/body weight ratios were reduced in animals fed Cd. Kidney histology showed predominantly Pb effects, i.e., intranuclear inclusion bodies and cloudy swelling. Ultrastructural evaluation of kidneys from Pb-treated animals disclosed nuclear inclusion bodies and mitochondrial swelling. Concurrent administration of Cd reduced total mean bone and kidney Pb levels by 50% and 60%, respectively, and this was associated with a decrease in kidney intranuclear inclusions. Cd exposure also reduced renal, femur, and liver concentrations of Fe by 33%, 43%, and 63%, respectively, decreased femur Zn

by 27%, but increased renal Zn by 20%. Administration of As produced mild swelling of tubule cell mitochondria, increased mean total renal Cu to 200% of control, and increased liver Fe by 44%. Dietary Pb produced increased urinary excretion of ALA and coproporphyrin. Dietary exposure to As caused increased urinary excretion of uroporphyrin and to a lesser extent coproporphyrin, whereas dietary Cd caused no significant changes in urinary levels of any of the porphyrins measured. Pb plus As produced an additive effect on coproporphyrin excretion but not that of ALA or uroporphyrin. They concluded that interactions between common toxic elements do occur and are characterized by alterations in both tissue trace metal levels and toxicity.

Shafigul et al., 2010 examined Arsenic and Cadmium in Food-chain in Bangladesh in an exploratory study. Arsenic and cadmium were measured in foods from Matlab, a rural area in Bangladesh, that is extensively affected by arsenic and the economy is agriculture-based. Raw and cooked food samples were collected from village homes (households, n=13) and analyzed to quantify concentrations of arsenic and cadmium using atomic absorption spectrophotometry. Washing rice with water before cooking reduced the concentration of arsenic in raw rice by 13–15%. Rice, when cooked with excess water discarded, showed a significant decrease in arsenic concentration compared to that cooked without discarding the water ($p<0.001$). In contrast, concentration of cadmium did not decrease in cooked rice after discarding water. Cooked rice with discarded water had significantly lower concentration of arsenic compared to raw rice ($p=0.002$). Raw rice had higher concentration of arsenic compared to raw vegetables ($p<0.001$); however, no such difference was found for cadmium. Compared to raw vegetables (e.g. arum), concentration of arsenic increased significantly ($p=0.024$) when cooked with arsenic-contaminated water. They concluded that the practice of discarding excess water while cooking rice reduces the concentration of arsenic but not of cadmium in cooked rice. However, water generally not discarded when cooking vegetables to avoid loss of micronutrients consequently retains arsenic. The results suggest that arsenic and cadmium have entered the food-chain of Bangladesh, and the cooking practices influence the concentration of arsenic but not of cadmium in cooked food.

Asagba (2010) investigated the comparative effect of water and food-chain mediated cadmium exposure in rats. The study compared the absorption and toxicity of Cadmium (Cd) administered via the food-chain and inorganic Cd administered in drinking water after 1 and 3

months exposure using rats as animal model. The food-chain was mimicked by exposing rats to diet containing Cd pre-exposed fish. The uptake of Cd by the rats after both mode of exposure was calculated by summing up the Cd burden in the liver and kidneys and was expressed in terms of % intake. The toxicity of Cd was assessed by monitoring biochemical indices of liver function in the plasma and liver. Regardless of the mode of exposure of the rats, the Cd load in the liver and kidney was significantly ($P < 0.05$) higher than the respective controls with the kidney having a significantly higher load than the liver after both periods of exposure. However irrespective of the mode of exposure, more Cd was accumulated in the liver and kidney of the 3 months exposed rats relative to those exposed for 1 month. The uptake of Cd by rats exposed to Cd via the food-chain for 1 and 3 months was significantly ($P < 0.05$) lower when compared to the corresponding water mediated Cd exposed rats, except for the liver after 3 months of exposure. The liver L-ALT activity of rats administered inorganic Cd in drinking water for 1 and 3 months was significantly ($P < 0.05$) lower as compared to controls. Parallel analysis of the plasma showed no significant ($P > 0.05$) difference in L-ALT activity between both groups after the same periods of exposure. The L-AST activity in the plasma of rats similarly exposed to Cd for 1 and 3 months was significantly ($P < 0.05$) higher as compared to controls with a corresponding reduction in the liver. Conversely no significant ($P > 0.05$) change was observed in plasma and liver L-ALT and L-AST activities after food-chain mediated exposure to Cd for 1 and 3 months in relation to their respective controls. His findings indicate that Cd incorporated in fish is more easily bioavailable, but less toxic relative to inorganic Cd salts at the end of 3 months of exposure in rats.

Asagba (2010) examined alteration in the activity of oxidative enzymes in the tissues of male Wistar albino rats exposed to cadmium. He assayed for the following oxidative enzymes [viz Aldehyde oxidase, AO (E.C. 1.2.3.1); Xanthine oxidase, XO (E.C. 1.2.3.2); Sulphite oxidase, SO (E.C.1.8.3.1.); and Monoamine oxidase, MO (E.C. 1.4.3.4)] in the liver and kidney. Male Wistar albino rats were administered 1, 2 and 4 mg Cd(2+)/kg body weight for one and three months. The activities of the oxidative enzymes were subsequently analyzed in the liver and kidney after both periods of exposure. His result showed that there was a dose dependent increase in liver and kidney Cd concentration in the test rats as compared to control after both periods of treatment with the liver retaining higher concentration of Cd than the kidney for each of the exposure dose. The oxidative enzymes were decreased in a dose dependent manner in the

liver and kidney after both periods of treatment. The percentage inhibition of these enzymes was less in the liver of rats treated with Cd for three months relative to the one month treated rats for each of the exposure dose. Conversely, the inhibition of the activities of these enzymes in the kidney of rats in all the treatment groups was more pronounced after three months relative to the trend in the one month treated rats. However, the activities of the oxidative enzymes were higher in the liver as compared to the kidney in all the treatment groups after both durations of Cd treatment.

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Equipment/Instruments

They include:

Spectrophotometer (model 721), Refrigerator (higher thermocol), pH meter (Jen way-3310), centrifuge (mse minor), Electronic weighing balance, electric water bath (techmel & techmel, U.S.A.), Electric weighing balance, Electric stabilizer, Mortar and pestle homogenizer, Burettes, Test tubes, Dissecting set, Pipettes, Bowls, Stop watch, Measuring cylinders, Beakers, Spatula, Syringe.

3.1.2 Chemicals/Reagents, kits and manufacturers

Chemicals/Reagents	Manufacturers
Ethanol (96%)	British Drug House, Poole, England
Ammonium Chloride	B.D.H, Poole, England
Dichloroindophenol	B.D.H, Poole England

Sodium Sulphite	May and Baker, Dagenham, England
Potassium Ferricyanide	May and Baker, Dagenham, England
Benzylamine	B.D.H, Poole England
Neutral Formaldehyde	B.D.H, Poole England
Methylene Blue	May and Baker, Dagenham, England
Ethylene diamine tetra acetic Acid (EDTA)	B.D.H, Poole England
Cadmium Chloride	British Drug House, Poole, England
Arsenic	British Drug House, Poole, England
Sodium chloride	British Drug House, Poole, England
Disodium hydrogen orthophosphate	British Drug House, Poole, England
Potassium dihydrogen phosphate	May and Baker, Dagenham, England
Sodium hydroxide	May and Baker, Dagenham, England
Chloroform	May and Baker, Dagenham, England

Kits	**Manufacturer**
Creatinine	Randox Laboratories Limited, UK
Urea	Teco Diagnostics, USA

All other reagents used in this study were of analytical grade

3.1.3 Fishes

For purpose of compounding diet for the experimental animal 100 catfishes were purchased from Delta State Government owned fish farm in obiaruku. The fishes were divided into 4 groups and left to acclimatize for 1 week.

- **Group A (Control) –** fishes in this group were housed in fresh water. This served as normal control group. The water was changed every 24 hours for 4weeks.
- **Group B (Arsenic) –** The water was contaminated with arsenic (0.4mg/100ml). The water was changed and re-contaminated every 24 hours for 4weeks.
- **Group C (Cadmium) –** The water was contaminated with cadmium (0.4mg/100ml). The water was changed and re-contaminated every 24 hours for 4weeks.

- **Group D (Arsenic + Cadmium) –** The water was contaminated with cadmium and arsenic (0.4mg/100ml of each contaminant). The water was changed and re-contaminated every 24 hours for 4 weeks.

All the fishes received normal feed for the duration after which they were killed, dried in an oven and used as protein source in the diet.

3.1.4 Preparation of diet

The diet was prepared as follow

1. **Protein:** African catfish prepared as described above served as source of protein and it made up 20% of the total diet.
2. **Carbohydrate:** corn starch served as the source of carbohydrate and it made up 40% of the total diet.
3. **Fats and oil:** vegetable oil served as source of fat and oil and it made up 10% of total diet
4. **Fibre:** laboratory cellulose served as the source as the source of fibre and it made 10% of the total diet.
5. **Sugar:** granulated refined sugar served as source of sugar and it made up 10% of the total diet.
6. **Vitamins and Minerals:** Vitamins and mineral mix (manufactured by Hebei Vsyong Animal pharmaceutical Co. Ltd, China) made up 10% of the total diet.

The mineral and vitamins were in the following proportion per 1kg of the mix.

Vitamin A	5,000,000I.U
Vitamin E	1500mg
Vitamin B2	2500mg
Vitamin B6	1000mg
Vitamin D3	500,000I.U
Vitamin B1	1000mg
Vitamin C	2000mg
Vitamin K3	250mg
Pantothenic acid	2000mg
Carnitine HCL	1500mg
Folic acid	50mg

Potassium Chloride	62.5g
Nicotinic acid	3000mg
Methionine	7500mg
Sodium chloride	62.5g
Calcium chloride	62.5g
Anhydrous glucosese	Q.S

3.1.5 Experimental Animals

Wistar strain of male albino rats (16) weighing between 100 to 150g were obtained from the college of Health Sciences, Delta State University, Abraka. They were housed in standard wooden cages in a room maintained at 25 ± 2 ^{0}C with a 12 hours light/dark cycle. Acclimatization was for two weeks before the commencement of experiments and the rats were given free access to standard laboratory feed (Top feeds Ltd., Sapele, Delta State) and water.

3.2.0 Methods

3.2.1 Experimental Design

Sixteen (16) male albino rats of Wistar strain were divided into four (4) groups with four (4) rats in each group.

- **Group A (Control)** - Animals in this group were fed daily for 12 weeks with normal diet (containing non-metal exposed fish as a source of protein) and water daily. This served as normal control group.
- **Group B (arsenic)** - Animals in this group were fed daily for 12 weeks with diet (containing arsenic contaminated fish) and water.
- **Group C (cadmium)** - Animals in this group were fed daily for 12 weeks with diet (containing cadmium contaminated fish) and water.
- **Group D (arsenic+cadmium)** - Animals in this group were fed daily for 12 weeks with diet (containing arsenic + cadmium contaminated fish) and water.

3.2.2 Collection of Tissues and Blood

After the appropriate treatment periods (12 weeks), the animals were weighed and sacrificed under chloroform anesthesia. The liver, kidney, brain, testis, prostrate and heart were dissected out, washed in normal saline, blotted individually on ash free filter paper, patted dry and weighed. The weighed tissues were stored in separate containers, labeled and immediately transferred to ice packs awaiting homogenization. Blood samples were taken from the animals by heart puncture and stored using lithium heparinized bottles.

3.2.3 Preparation of Samples

Ten percent homogenate of the liver, heart, prostrate, testis, brain and kidney were prepared using normal saline (0.9% NaCl) under cold conditions. The homogenates were centrifuged at 5000 g for 10 minutes and the supernatants obtained were subsequently stored in refrigerator in preparation for analysis. The blood samples collected in lithium heparin bottles were centrifuged at 5000 g for 10 minutes to obtain the plasma and stored in the refrigerator in preparation for analysis.

3.3.0 Biochemical Assays

3.3.3 Assay for Aldehyde Oxidase (AO) Activity

The activity of the enzyme in the tissue was determined by the method of Omarov et al (1999).

Principle

The activity was monitored based on the decrease in absorbance at 600nm which is resultant of the oxidation of benzaldehyde to benzoate using 2, 6 –Dichloroindophenol as electron donor.

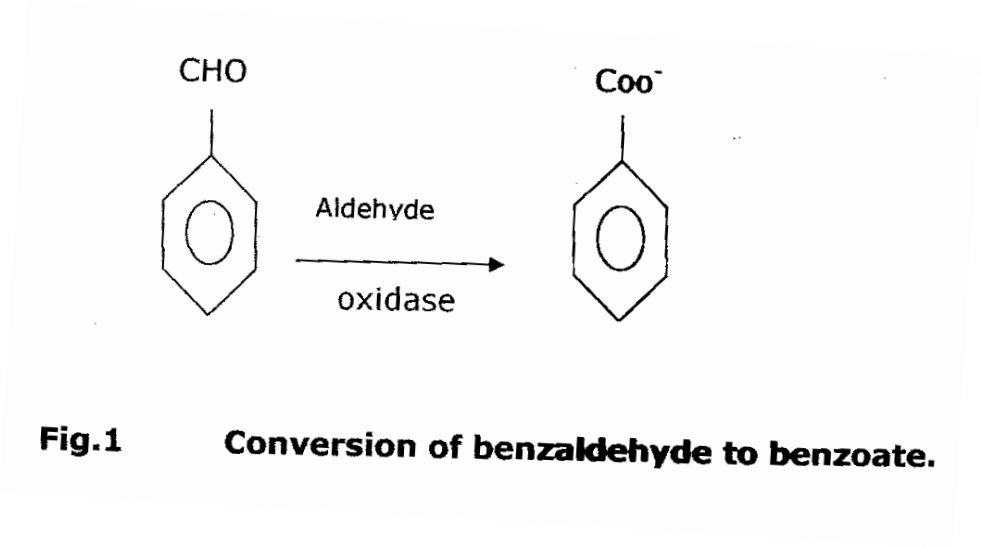


Fig.1 Conversion of benzaldehyde to benzoate.

Procedure

The reaction was initiated by the addition of 0.1ml benzaldehyde (1mM), which was used as the substrate into a test tube. Thereafter, 1.7ml of 0.2M phosphate butter (pH 7.8), 0.4ml of Ammonium Chloride solution (4mM), 0.4ml of EDTA (1mM), 0.4ml of 0.002% 2,6 - Dichloroindophenol and 0.2ml of the supernatant were added to the test tube. The mixture was quickly transferred into a curvette. The decrease in absorbance was read at 600nm against reagent blank at every 30 seconds.

Calculation

Aldehyde oxidase activity was estimated using the formula:

Activity (micromoles benzoate/g wet weight) =

$$\frac{\Delta A \; X \; V_T}{\Sigma \; X \; V_S \; X \; X^1}$$

Where

V_T = Total volume of reaction mixture

V_S = Volume of sample used

ΔA = Change in absorbance per minute at 600nm

X^1 = Weight of tissue in reaction mixture

Σ= Molar extinction coefficient (19,100 m^{-1}).

One unit of the enzyme is the amount of the enzyme that produces one micromole of benzoate per minute.

3.3.4 Assay for Monoamine Oxidase (MAO) Activity

The method used for the assay of monoamine oxidase activity was proposed by Tabor et al (1954)

Principle

$R\text{-}CH_2\text{-}NH_2\text{-}O_2 \xrightarrow{MAO} RCHO + NH_3 + H_2O_2$

Aldehyde ammonia hydrogen peroxide

Procedure of Assay

The assay was carried out by adding 2.5ml of 0.2M phosphate buffer (pH 7.8), 1ml of 0.1M benzylamine and 0.2ml of the supernatant into labeled test tubes. A blank was similarly prepared but it contained 0.2ml of water instead of sample. The absorbance was immediately read at 340nm against the reagent blank and recorded every 30 seconds for one minute.

The activity of monoamine oxidase is given in units per gramme tissue. One unit of the enzyme is defined as the amount of the enzyme that will produce one micromole of benzaldehyde per minute.

Calculation

$$\frac{\Delta A \; X \; V_T}{\Sigma \; X \; V_S \; X \; X^1}$$

Where

V_T = Total volume of reaction mixture

V_S = Volume of sample used

ΔA = Change in absorbance per minute

X^1 = Weight of tissue in reaction mixture

Σ= Molar extinction coefficient (12,800m^{-1}).

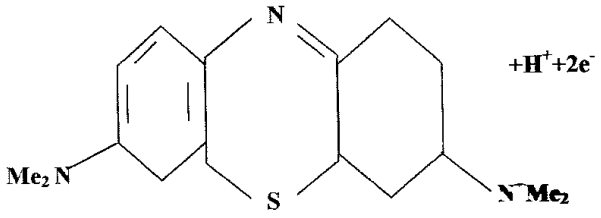
3.3.5 Assay for Xanthine Oxidase (XO) Activity

The enzyme activity was determined by the method of Dixon and Thurlow (1924)

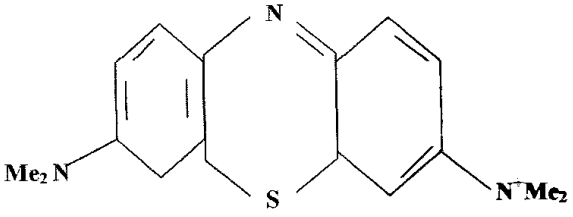
Principle

Xanthine oxidase catalyses the conversion of methylene blue to the reduced colourless form. The change in the colour of methylene blue has been associated with the following structural change.

33



Blue (Resonance across nitrogen between rings)



Colourless (No resonance across nitrogen between rings).

Procedure:

The reaction was initiated by the addition of 1ml of 0.5% neutral formaldehyde into a test tube. Thereafter, 1ml of 0.02% methylene blue solution, 1ml of sample and two (2) drops of

liquid paraffin were also added to the test tube. Then, the time taken for the discoloration of ethylene blue was recorded. The blank test tube was similar to the 'test' test tube except the absence of 0.02% methylene blue solution. The function of liquid paraffin is to prevent atmospheric oxidation

$$\textit{Enzyme activity} = \frac{1}{\textit{Time taken for methylene blue to change colourless}}$$

The activity of xanthine oxidase is expressed as $min^{-1}g^{-1}$ wet tissue.

3.3.6 Assay For Sulphite Oxidase (SO) Activity

Sulphite oxidase activity was determined by the reduction of ferricyanide (Macleod et al., 1961). One unit of the enzyme is defined as the amount of the enzyme that reduces one micromole of ferricyanide per minute.

Principle

$Na_2SO_3 + O_2 + H_2O \longrightarrow Na_2SO_4 + H_2O_2$

Sulphite oxidase catalyses the oxidation of sulphite to sulphate with ferricyanide as electron acceptor.

Procedure of Assay

To each test tube, 2.5ml of phosphate buffer (0.5M) was added followed by the addition of 0.5ml of potassium ferricyanide (0.005%), 0.6ml of sodium sulphite (1mM) solution, 0.2ml of EDTA (1mM) and 0.2ml of homogenate supernatant. Immediately, the mixture was carefully transferred into a curvette and the absorbance read at 420nm against reagent blank.

Calculation

$$\frac{\Delta A \; X \; V_T}{\Sigma \; X \; V_S \; X \; X^1}$$

Where

V_T = Total volume of reaction mixture

V_S = Volume of sample used

ΔA = Change in absorbance per minute at 420nm

X^1 = Weight of tissue in reaction mixture

Σ= Molar extinction coefficient (1.02 X 10^4 m^{-1} cm^{-1}).

3.3.7. Urea Concentration Estimation Using Randox Assay Kit (Urease-Berthelot Method).

Principle (Weatherburn, 1967)

Urea in serum is hydrolysed to ammonia in the presence of urease. The ammonia is then measured photometrically by Bethelot's reaction.

Urea + H_2O $\xrightarrow{urease}$ $2NH_3$+ CO_2

NH_3 + hypochlorite + phenol ⟶ indophenols (blue compound)

Reagent Composition

Contents	Concentration of Solutions
CAL: Standard Calcium	80.65mg/dl (13.42mmol/l)
R1: EDTA	116mmol/l
Sodium nitroprusside	6mmol/l
Urease	1g/l
R2: Phenol (diluted)	120mmol/l
R3: Sodium hypochlorite (diluted)	27mmol/l
Sodium hydroxide	0.14N

Procedure:

Ten microliter each of distilled water, sample, and standard (Cal) were pipetted into 'blank', 'Test', and 'Standard' test tubes respectively. Thereafter 100 µl of Reagent 1 was added to the three test tubes, mixed and incubated at 37^0C for 10 minutes. Then 250 µl of Reagent 2

and 3 was added to the three test tubes, mixed immediately and incubated at 37^0C for 15 minutes. Absorbance of the sample (A_{Sample}) and standard ($A_{Standard}$) were read against the blank at 546nm.

Calculation:

$$\text{Urea concentration (mmol/l)} = \frac{A_{Sample}}{A_{Standard}} \times \frac{13.3}{1}$$

Normal value (Mackay and Mackay, 1927) 1.7-9.1 mmol/l (10-55mg/dl

3.3.8 Determination of Serum Creatinine Using Randox Assay Kit.

Principle (Bartels and Bohmer, 1972): Creatinine in alkaline solution reacts with nicric acid to form a coloured complex. The amount of the complex formed is directly proportional to the creatinine concentration.

Reagent Composition:

Contents	Concentration of Solutions
CAL: Standard	2.06mg/dl (182mmol/l)
RIa: Picric Acid	35mmol/l
RIb: Sodium Hydroxide	0.32mol/l
Working Reagent	Equal volumes of R1a + R1b

Procedure:

Working reagent (1.0ml) was added to two test tubes labeled, ‘standard’ and ‘sample’. Standard solution (0.1ml) was then added to the ‘standard’ test tube while the sample (0.1ml) was added to the ‘sample’ test tube. They were mixed and after 30 seconds the absorbance A_1 of the standard and sample were read. Exactly 2 minutes later, the absorbance A_2 of standard and sample were again read at 492nm.

Calculation:

Manual calculation:

$A_2 - A_1 = \Delta A$ sample or ΔA standard

Concentration of Creatinine in serum:

$\frac{\Delta A\text{sample}}{\Delta A\text{standard}} \times 2.06\text{mg/dl}$

Normal Values: 0.5-1.1 mg/dl

3.7 Statistical Analysis

Results are presented as Mean ± SEM. Difference in the means were analyzed using Analysis of Variance (ANOVA) multiple comparison test and Least Significant Test (LSD). Statistical significance was given as $P<0.05$ as indicated in the results.

CHAPTER FOUR
RESULTS

4.1: Effect of Food chain Mediated Metal Exposure on Weight Gain

The separate and combined effect of arsenic and cadmium on body weight gain and organ/body weight ratio of rats is presented in Table 1. There was no significant difference in body weight gain of rats administered with the combined dose but a significant increase in body weight gain was observed in rats administered with arsenic and cadmium separately relative to control. Thus, this study shows that arsenic and cadmium has no influence on body weight gain when combined.

The liver, testis, heart, and brain /body weight ratio of rats was not significantly different from control after combined and separate treatment with arsenic and cadmium. On the other hand the organ/body weight ratio for kidney was significantly increased after separate and combined treatment with arsenic and cadmium. Similarly the prostate/body weight ratio of rats was significantly increased in rats administered either cadmium or a combination of cadmium and arsenic. Conversely there was significantly decreased prostate/body weight ratio in rats administered arsenic relative to control.

Table 1: Body Weight Gain and Organ/Body Weight Ratio of Rats Exposed To Cadmium and Arsenic via the Food Chain

Parameter	Control	Arsenic	Cadmium	Arsenic + Cadmium
Body weight gain	77.50±7.59[a]	39.27±11.65[b]	37.62±5.85[c]	80.07±5.39[a]
Liver/bodyweight ratio	0.034±0.20[a]	0.0350±0.05[a]	0.0305±0.28[a]	0.0339±0.33[a]
Kidney/ bodyweight ratio	0.006±0.03[a]	0.008±0.04[b]	0.007±0.02[d]	0.007±0.01[c]
Testis/ bodyweight ratio	0.012±0.60[a]	0.014±0.17[a]	0.013±0.08[a]	0.011±0.06[a]
Heart/ bodyweight ratio	0.003±0.20[a]	0.004±0.02[a]	0.0037±0.02[a]	0.004±0.01[a]
Prostrate/ bodyweight ratio	0.004±0.05[a]	0.003±0.05[b]	0.005±0.028[c]	0.006±0.08[c]
Brain/ bodyweight ratio	0.008±0.08[a]	0.010±0.03[a]	0.008±0.03[a]	0.008±0.01[a]

Values are expressed in Mean ± Standard error of Mean (SEM) N=4, significance at ($P<0.05$), values not sharing a common superscript in same row differs at $P<0.05$ using analysis of variance (ANOVA).

4.2: Effect of Food Chain Mediated Exposure on Monoamine Oxidase

Table 2 presents the effect of arsenic, cadmium and combination of both metals on tissue monoamine oxidase activity of experimental rats. The liver MAO activity was not significantly different from control in rats treated separately with arsenic, cadmium and combination of both.

Similarly, the kidney, testis, prostate, and brain MAO activities were not significantly different from control in rats treated with arsenic, cadmium and a combination of both metals. Thus the study shows that the MAO activity was not influence by metals in these organs.

The heart MAO activity of rats administered arsenic was significantly decreased relative to control. Conversely while this parameter was significantly increased in rats administered a combination of both metal, there was no significant difference in rats administered cadmium.

Table 2: Monoamine Oxidase Activity In Rats Exposed To Cadmium And Arsenic

Parameter	Control	Arsenic	Cadmium	Arsenic + Cadmium
Liver (units/g tissue)	22.33±1.58 [a]	24.64±0.82 [a] (10.35%)	23.86±2.51 [a] (6.88%)	23.55±1.48 [a] (5.56%)
Kidney(units/g tissue)	32.97±2.08 [a]	28.41±1.37 [a] (-13.8%)	29.62±2.15 [a] (-10.139%)	33.73±2.56 [a] (-10.139%)
Testis (units/g tissue)	45.61±.4.99 [a]	46.25±.5.69 [a] (1.38%)	35.92±.1.04 [a] (-21.25%)	40.92±4.93 [a] (-7.66%)
Heart (units/g tissue)	50.77±2.96 [a]	46.88±6.09 [b] (7.668%)	51.32±4.33 [a] (1.08%)	60.43±2.33 [b] (19.02%)
Prostrate (units/g tissue)	41.04±4.24[a]	39.22±3.35 [a] (-4.44%)	37.34±3.10 [a] (-9.02%)	38.51±5.26[a] (-6.16%)
Brain (units/g tissue)	44.80±2.05[a]	41.66±3.55 [a] (-7.01%)	48.94±1.21 [a] (9.23%)	46.59±2.78 [a] (3.99/%)

Values are expressed in Mean ± Standard error of Mean (SEM) N=4, significance at ($P<0.05$), values not sharing a common superscript in same row. Differs at $P<0.05$ using analysis of variance (ANOVA).

4.3: Effect of Food Chain Mediated Exposure on Sulphite Oxidase

Table 3 represents the effect of arsenic, cadmium and combination of both metals on tissue sulphite oxidase activity of experimental rats. There was no significant difference in the sulphite oxidase activity in liver and testes as compared to control in rats treated with arsenic, cadmium and cadmium + arsenic.

Similarly, Kidney sulphite oxidase was not significantly different from control in rats treated with arsenic, cadmium or arsenic plus cadmium. Conversely, the heart and brain sulphite oxidase activity was significantly increased in rats administered arsenic, cadmium or combination of both metals.

The prostate sulphite oxidase activity was not significantly different from the control in rats administered cadmium or cadmium plus arsenic, but was significantly increased in rats treated separately with arsenic.

Table 3: Sulphite Oxidase Activity in Rats Exposed to Cadium and Arsenic

Parameter	Control	Arsenic	Cadmium	Arsenic + Cadmium
Liver (units/g tissue)	17.69±0.22 [a]	19.38±0.81 [a] (9.56%)	18.82±0.38 [a] (6.01%)	19.33±0.63 [a] (9.25%)
Kidney (units/g tissue)	17.30±0.16 [a]	16.29±0.39 [a] (-5.80%)	18.82±0.58[a] (-0.08%)	16.96±0.39 [a] (-1.98%)
Testis (units/g tissue)	19.87±0.81 [a]	18.23±1.37 [a] (-8.26%)	18.72±0.34 [a] (-5.79%)	18.33±0.81 [a] (-7.76%)
Heart (units/g tissue)	14.46±1.38 [a]	17.62±0.72[b] (21.85%)	18.70±0.70[b] (29.32%)	17.81±0.63[b] (23.22%)
Prostrate (units/g tissue)	17.83±0.58 [a]	20.34±0.53[b] (14.08%)	16.49±0.25[a] (-7.49%)	17.03±0.32[a] (-4.46%)
Brain (units/g tissue)	13.55±0.36 [a]	19.13±2.41[b] (41.13%)	18.72±0.06[b] (38.15%)	16.96±0.34[c] (25.13%)

Values are expressed in Mean ± Standard error of Mean (SEM) N=4, significance at ($P<0.05$), values not sharing a common superscript in same row differs at $P<0.05$ using analysis of variance (ANOVA). Figures in parenthesis represent percentage changes from control

4.4: Effect of Food Chain Mediated Exposure on Aldehyde Oxidase

Table 4 represents the effect of arsenic, cadmium and combination of both metals on tissue Aldehyde oxidase activity of experimental rats. The liver and brain aldehyde oxidase activities were not significantly different from control in rats treated with arsenic, but was significantly increased in rats treated with cadmium or combination of cadmium and arsenic.

On the other hand kidney AO activity was not significantly different from control in rats treated with arsenic, but was significantly decreased in rat administered cadmium and cadmium plus arsenic. Similarly the heart AO activity was significantly decreased after exposure to cadmium, arsenic or combination of both metals. No significantly difference was observed in the activity of AO in the prostate and testis after separate or combined exposure to both metals.

Table 4: Aldehyde Oxidase Activity In Rats Exposed To Cadmium And Arsenic

Parameter	Control	Arsenic	Cadmium	Arsenic + Cadmium
Liver	23.99±1.97 [a]	23.05±2.37 [a] (-3.90%)	0.68±1.37[b] (27.85%)	30.37±1.76[b] (26.59%)
Kidney	28.93±3.30 [a]	27.21±0.99 [a] (-21.51%)	25.40±0.90[b] (-12.21%)	25.60±1.57[b] (-11.52%)
Testis	8.22±0.86 [a]	12.13±3.97 [a] (47.55%)	7.33±0.21 [a] (-10.88%)	8.87±0.65 [a] (7.89%)
Heart	8.07±0.52 [a]	7.44±0.93[b] (-7.77%	5.83±0.13[c] (-27.75%)	6.68±0.29[b] (-17.180%)
Prostrate	6.19±0.27 [a]	7.12±0.58 [a] 14.85%)	6.56±0.61 [a] (5.90%)	6.50±0.08 [a] (4.90%)
Brain	1.58±0.12 [a]	3.95±1.03 [a] (150.34%)	6.01±0.15[b] (280.13%)	6.01±0.19[b] (280.13%)

Values are expressed in Mean ± Standard error of Mean (SEM) N=4, significance at ($P<0.05$), values not sharing a common superscript in same row differs at $P<0.05$ using analysis of variance (ANOVA). The activity of aldehyde oxidase is given as µmol benzoate/g wet weight tissue.

4.5: Effect of Food Chain Mediated Exposure on Xanthine Oxidase

The effect of arsenic, cadmium and combination of both metals on tissue xanthine oxidase activity of experimental rats is presented in Table 5. The liver xanthine oxidase (XO) activity was not significantly different from control in rats treated with arsenic, but was significant decreased in rats treated with cadmium. Conversely administration of both metals significantly increased liver XO activity relative to control.

The kidney, testis, prostrate and brain XO activity was not significantly different from control in rats treated with arsenic, cadmium and a combination of cadmium and arsenic. The heart XO activity of rats administered arsenic was significantly decreased arsenic was significantly decreased compared to control.

On the other there was no significant difference in heart XO activity in rats administered cadmium plus arsenic, but this parameter was significantly increased in those separately treated with cadmium.

Table 5: Xanthine Oxidase Activity in Rats Exposed To Cadmium And Arsenic

Parameter	Control	Arsenic	Cadmium	Arsenic + Cadmium
Liver	0.41±0.04 [a]	0.41±0.04 [a] (0%)	0.29±0.024[c] (-29.99%)	0.83±0.16[b] (99.97%)
Kidney	0.37±0.04 [a]	0.58±0.14 [a] (55.54%)	0.45±0.04 [a] (22.21%)	0.41±0.04 [a] (11.12%)
Testis	0.20±0.00 [a]	0.17±0.01 [a] (-11.3%)	0.22±0.01 [a] (12.5%)	0.20±0.01 [a] (0.00%)
Heart	0.31±0.020 [a]	0.27±0.03[b] (-10.65%)	0.41±0.04[c] (33.34%)	0.35±0.05 [a] (13.34%)
Prostrate	0.41±0.04 [a]	0.33±0.058 [a] (-19.91%)	0.35±0.05 [a] (-14.90%)	0.29±0.02 [a] (-30.04%)
Brain	0.28±0.07 [a]	0.54±0.15 [a] (88.4%)	0.39±0.06 [a] (37.66%)	0.35±0.05 [a] (23.2%)

Values are expressed in Mean ± Standard error of Mean (SEM) N=4, significance at ($P<0.05$), values not sharing a common superscript in same row differs at $P<0.05$ using analysis of variance (ANOVA). The activity of xanthine oxidase is presented in min/g wet weight.

4.6: Effect of Food Chain Mediated Exposure on Plasma Creatinine and Urea Concentrations

Table 6 presents the effect of arsenic, cadmium and combination of both metals on plasma creatinine and urea concentration of experimental rats the creatinine concentration in the plasma of rats was not significantly different from the control in rats treated with arsenic and a combination of cadmium and arsenic but was significantly increased in rats treated with cadmium. The urea concentration in the plasma of rats was not significantly different from the control in rats treated with arsenic, cadmium or the combination of both metals.

Table 6: Creatinine and Urea in Plasma of Metal Treated Rats

	Creatinine mg/dl	Urea mg/dl
Control	2.20±0.025[a]	17.46±1.69 [a]
Arsenic	2.34±0.11[a]	18.84±0.85 [a]
	(6.58%)	(7.88%)
Cadmium	2.57±0.08[b]	19.52±0.29 [a]
	(17.07%)	(11.76%)
Cadmium + arsenic	2.30±0.06[a]	19.79±0.46 [a]
	(4.45%)	(13.34%)

Values are expressed in Mean ± Standard error of Mean (SEM) N=4, significance at (P<0.05), values not sharing a common superscript in same column differs at P<0.05 using analysis of variance (ANOVA)..

CHAPTER FIVE

DISCUSSION, CONCLUSION, AND CONTRIBUTION TO KNOWLEGDE

5.1 Discussion

In this study, the effects of food-chain mediated exposure to Cd, As and combination of both metals on weight gain, organ/body weight ratio, tissue oxidative enzymes and plasma urea and creatinine levels in rats were evaluated.

There was no significant difference in body weight gain of rats administered with the combined dose but a significant increase in body weight gain was observed in rats administered with arsenic and cadmium separately relative to control. Thus, this study shows that arsenic and cadmium has no influence on body weight gain when combined. This result contrasts that of Mahaffey et al. (2008) who studied the effects of concurrent exposure of lead, cadmium, and arsenic on tissue metal accumulation in rats in which Cd and As reduced weight gain even when differences in food intake were taken into account, and administration of both Cd and As depressed weight gain more than did either metal alone.

Some of the morphological parameters that have often been used for the assessment of toxicity of chemicals are changes in body weight gain and organ/body weight ratio (Timbrell, 1991). Thus the significantly decreased body weight gain of rats administered Cd and As separately in relation to control (Table 1) may be an indication of the adverse effect of both metals. This finding is not surprising as both metals have been associated with disruption of gastrointestinal system (Ghariani et al., 2001).

The liver, testis, heart, and brain /body weight ratio of rats was not significantly different from control after combined and separate treatment with arsenic and cadmium. On the other hand the organ/body weight ratio for kidney was significantly increased after separate and combined treatment with arsenic and cadmium. The alteration of the organ/body weight ratio observed in rats administered Cd, As or their combination is also an indication of toxicity.

The oxidative enzymes examined in this study which are involved in the oxidation of xentbiotics are aldehyde, xanthine, and sulphite oxidases, all of which are molybdenum and haem containing soluble enzymes that are present in the liver and other tissues (Rang et al., 2001; Beedham, 2002; Hillle, 2005). Monoamine oxidase is also important as it is involved in

the biotransformation of aromatic monoamines, including classical neurotransmitters such as serotonin, adrenalin, histamine and dopamine.

Monoamine oxidase is involved in the metabolism of some dietary constituents (e.g. tyramine, tryptophan and other amines and amine precursors) as well as many drugs (e.g. sympathomimetics, opiates, reserpine and caffeine) (Murphy and Kalin, 1980). Similarly the molybdenum hydroxylases, Aldehyde oxidase and Xanthine oxidase both play important roles in the metabolism of many exogenous and endogenous compounds. The cytosol of various mammals also exhibits a significant reductive activity toward nitro, sulfoxide, N-oxide and other moieties catalyzed by Aldhyde oxidase (Kitamura et al., 2006). The conventionally accepted role of Xanthine oxidase is purine catabolism, in which it catalyzes the oxidation of hypoxanthine to xanthine, then to uric acid (Kitamura et al., 2006). Sulphite oxidase, another molybdoprotein, is involved in the oxidation of endogenous sulphite arising from the degradation of sulphur amino acids (Cohen et al., 1973).

In this study, liver xanthine oxidase (XO) activity was not significantly different from control in rats treated with arsenic, but was significant decreased in rats treated with cadmium. Conversely administration of both metals significantly increased liver XO activity relative to control. On the other there was no significant difference in heart XO activity in rats administered cadmium plus arsenic, but this parameter was significantly increased in those separately treated with cadmium. Exposure to non-essential metals such as cadmium and arsenic have been reported to be one of the factors, which might influence the activities of xenobiotic metabolizing enzymes (Moore, 2004). One of the main defence mechanisms by animals to avoid concentrating foreign compounds is to enzymatically transform them to less harmful excretable compounds. This biotransformation process occurs mostly in the hepatic tissues and, to a lesser extent, in some extra hepatic tissues (Timbrell, 1991). Oxidation reactions are probably the most common phase I reactions in xenobiotic biotransformation, and for these processes, a group of non-specific, cytochrome P-450-dependent mixed function oxidases (MFO) are required. Studies have shown that exposure to cadmium decreased cytochrome P-450 activity in both fish and rodents (Plewka et al., 20004; Henczova et al., 2006), and by extention the activity of AO. This thus, explains the decreased activity of AO in the liver and heart of rats fed fish contaminated with Cd.

The activities of the oxidative enzymes were tissue normalized, because Cd exposure is known to affect tissue weight. On this basis, there was observed decreases in oxidative enzymes in the liver, kidney and testes, which may be an indication that Cd interfers with the activities of these enzymes. This also agrees with the report that cadmium induces toxicity on target tissues by interfering with enzymatic processes (Gills et al., 1991). Timbrell (1991) had reported that the inhibition of enzymes by Cd can be linked to the displacement of essential metals cofactors from the enzyme active site or the formation of covalent bonds by cadmium with sulphdryl and other groups essential for the enzyme action. The Cd-induced inhibition of the oxidative enzymes observed in this study may thus be by any of these effects.

The liver MAO activity was not significantly different from control in rats treated separately with arsenic, cadmium and combination of both. Similarly, the kidney, testis, prostate, and brain MAO activities were not significantly different from control in rats treated with arsenic, cadmium and a combination of both metals. Thus the study shows that the MAO activity was not influence by metals in these organs. MO inhibition is accompanied by marked changes in the activity of the organism to some dietary constituents (e.g. tyramine, tryptophan and other amines and amine precursors) as well as many drugs (Murphy and Kalin, 1980). Cadmium administered directly to rats has been shown to alter the activity of this enzyme (Asagba et al., 2010). The lack of effect in this study suggest that the food-chain mediation may have reduced the level of the cadmium such that is was not affective on this enzyme.

Like MAO, the molybdenum hydroxylases AO and XO both play important roles in the metabolism of many exogenous and endogenous compounds. They exhibit oxidative activity towards various heterocyclic compounds and aldehydes (Kitamura et al., 2006). The conventionally accepted role of XO is purine catabolism, in which it catalyses the oxidation of hypoxanthine to xanthine, then to uric acid (Kitamura et al., 2006). SO, another molybdopterin is involved in the oxidation of endogenous sulphite arising from the degradation of sulphur amino acids (Cohen et al., 1973). Thus, the decreases observed in the activities of these oxidative enzymes would not only affect their contribution towards the detoxification of xenobiotics but may also affect other aspect of rat metabolism, for instance the decrease in SO would invariably affect the metabolism of sulphur-containing amino acids. This in turn may affect the metabolism of glutathione and proteins such as metallothionein, which are essential free radical scavengers, thus this may aggrevate the toxicity of cadmium and arsenic in the rat.

The activity of MAO in the brain of rats used in this study was not significantly different across the experimental groups (Table 2). Available reports indicate that the brain has the capacity to induce the synthesis of MT (Aschner 1996; Minami et al., 2010), although this molecule is not highly induced in the brain relative to other tissues. Thus, the induction of MT in the brain and distant tissues may partly account for the lack of significant change in the activities of brain MAO. Methalothioneins have the capacity to sequester heavy metals thus rendering them non-toxic. They are also efficient receptor of free radicals (Aschner 1996).

The creatinine concentration in the plasma of rats was not significantly different from the control in rats treated with arsenic and a combination of cadmium and arsenic but was significantly increased in rats treated with cadmium. Similarly, the urea concentration in the plasma of rats was not significantly different from the control in rats treated with arsenic, cadmium or the combination of both metals (Table 6). Serum urea and creatinine levels in animals are indicative of muscular wastage (Fashina, 1991). Higher urea and creatinine values may be brought about by the inadequacy or unavailability of the dietary protein, poor digestibility or inefficient utilization of the protein (Adesehinwa and Ogunmodede, 2004), but this was not reflected in the overall result in this study. Urea is the main nitrogenous end product arising from the catabolism of amino acids that are not used for biosynthetic roles in mammals and is an indicator of muscular wastage in animals (Adesehinwa, 2004). However, it is noteworthy that alterations in serum urea or creatinine is indicative of kidney dysfunction. Thus the lack of significant difference in serum creatinine in rats exposed to both metals via the food-chain is a likely indication of a lack of kidney dysfunction. Renal damage has been reported to occur when rats are exposed to cadmium and other heavy metals (Alvarez et al., 2007).

Finally, it is worth noting that the pattern of result obtained showed that each metal appears to influence the effect of the other. However, these influences depend not only on the tissue but also the parameter involved.

5.2 Conclusion

Cd and As have long been shown to be dangerous to human health since they can bioaccumulate in tissues. In this study, the effects of food-chain mediated exposure of Cd, As and combination of both metals on weight gain, organ/body weight ratio, tissue oxidative enzymes and plasma urea and creatinine levels in rats were evaluated. The results obtained indicate that cadmium and arsenic when administered separately significantly decreased body

weight gain of rats. Both metals and their combination also decreased kidney/body weight ratio of rats. Alterations of the oxidative enzymes was also observed in rats administered both metals and their combinations. Thus the findings of the study indicate that these metals were toxic when administered via the food-chain since they can affect the ability of the rats to use the oxidative enzymes for biotransformation.

5.3 Contribution to Knowledge

This study has been able to show the mechanism of activity of oxidative enzymes by which cadmium and arsenic exert their toxic potentials along the food chain.

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APPENDIX I

PREPARATION OF REAGENTS/CHEMICALS

Preparation of Phosphate Buffer, pH (7.4)

Anhydrous dibasic sodium phosphate (NaH_2PO_4) 11. 42g and 2.18g of anhydrous monobasic potassium phosphate (KH_2PO_4) were weighed, dissolved in distilled water by stirring continuously. The pH of the solution was measured using a pH meter and adjusted to 7.4 by adding IN NaOH dropwise before making the solution up to the required volume with distilled water. The solution was stored in a refrigerator until required.

Preparation of Coloured Reagent

Dimitrophenylhydrazine [$(NO_2)_2C_6H_3NHNH_2$] 0.0396g was dissolved in 200ml of 1 N HCL, and stored in the refrigerator until required.

Preparation of 0.9% Normal Saline

NaCl salt (0.9g) was weighed and placed in a beaker, where it was dissolved by adding 80ml of distilled water, stirred properly and then made up the volume to 100ml before storing in a refrigerator until required.

Preparation of IN NaOH solution

Sodium hydroxide pellets (40g) was weighed and placed in a beaker where it was dissolved with 20ml of distilled water by stirring continuously and then diluted to 1000ml (1 litre), by making the volume up with distilled water. It was then stored in a refrigerator until required.

PREPARATION OF ALDEHYDE OXIDASE (AO) REAGENTS

(i) **0.002% 2, 6- Dichloroindophenol (0.002g)**

2,6- dichloroindophenol (0.002g) was dissolved in 90ml of distilled water and then made up to 100ml of distilled water.

(ii) 4MmM Ammonium Chloride

NH_4Cl (0.02g) was dissolved in 90ml of distilled water and then made up to 100ml.

PREPARATION OF MONOAMINE OXIDASE (MAO) REAGENTS

(i) 0.2M phosphate buffer pH 7.8

Na_2HPO_4 (2.1g) and 3.35g KH_2PO_4 was dissolved in 900ml of distilled water and the pH was adjusted with 0.1M HCl and then made up to 1000ml.

(ii) 0.1M Benzylamine

Benzylamine (21.4ml) was dissolved in 190ml of distilled water and then made up to 200ml with distilled water.

PREPARATION OF XANTHINE OXIDASE (XO) REAGENTS

(i) o.5% Neutral Formaldehyde

Formaldehyde (o.5ml) was dissolved in 95ml of distilled water and mixed properly.

(ii) 0.02% Methylene blue Solution

Methylene blue (0.02g) was dissolved in 80ml of distilled water and made up to 1000ml.

PREPARATION OF SULPHITE OXIDASE (SO) REAGENTS

(i) 0.5M phosphate buffer pH 7.8

Sodium dihydrogen orthophosphate NH_2PO_4 4.26g and dipotassium dihydrogen orthophosphate 2.72g were dissolved in 900ml of distilled water and the pH was adjusted with 0.1M HCl and then made up to 1000ml with distilled water.

(ii) 1mM SODIUM Sulphite

Sodium Sulphite (0.025g) was dissolved in 190ml of distilled water and then made up to 200ml with distilled water.

(iii) 1mM EDTA

EDTA 0.116g was dissolved in 390ml distilled water and this was made up to 400ml with distilled water

(iv) 0.005% Potassium Ferricyande

Potassium ferricyande (0.005g) was dissolved in 90ml of distilled water and then made up to 100ml with distilled water

(v) O.IM HCl

Concentrated HCl (2.57ml) was dissolved in 100ml of distilled water.

APPENDIX II

STATISTICAL SYMBOLS/FORMULAE

1. Mean $(\overline{X}) = \frac{\Sigma x}{n}$

2. Standard Deviation (S.D) = $\sqrt{\frac{\Sigma(x-x)}{n}}$

3. Analysis of Variance (ANOVA)

Step 1 = Correction Factor (CF) = $\frac{(\Sigma\Sigma x)^2}{N}$

Where N = total numbers of observation

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Step 2 = Total Sum of Squares (TSS)

$TSS = \Sigma\Sigma x^2 - CF$

Step 3 = Treatment Sum of Squares (TrSS)

$$TrSS = \left[\frac{\Sigma(\text{Treatment Totals})^2}{\text{No of Replicates}}\right] - CF$$

Step 4 = Error Sum of Square (ErSS)

ErSS = TSS – TrSS

Step 5 = Mean Square (MS)

$$MS = \frac{\text{Sum of Square (SS)}}{\text{Degree of Freedom (DF)}}$$

(a) Mean Square Treatment (MS_{Tr}) = $\frac{\text{TrSS}}{\text{DF}_{\text{Tr}}}$

(b) Mean Square Error (MS_{error}) = $\frac{\text{ErSS}}{\text{DF error}}$

Step 6 = Degree of Freedom (DF)

Total DF = N -1

N = Total number of treatments – 1

Error DF = Total DF – Treatment DF

Step 7 = F – value = $\frac{\text{Mean Square Treatment}}{\text{Mean Square Error}}$