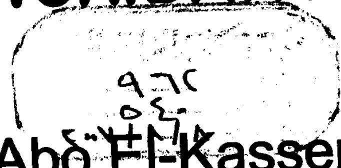
MINIA UNIVERSITY
FACULTY OF AGRICULTURE
DEPARTMENT OF AGRICULTURAL CHEMISTRY

BIOCHEMICAL STUDIES ON SOME NATURAL ANTIOXIDANTS

BY



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B.Sc. Agricultural Sciences, June, 2003
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THESIS

Submitted for partial fulfillment of the Requirements for
The M.Sc. Degree in Agricultural Chemistry

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ABBREVIATION

Antioxidant enzymes

CAT	Catalase
SOD	Superoxide dismutase
GP_X	Glutathione peroxide
GST	Glutathione -S- transferase
GR	Glutathione reductase
LPO	Lipid peroxidase

ROS	Reactive oxygen species
MDA	Malondialdyde
EDTA	Ethylene diamine tetra acetic acid
GSH	Reduced glutathione
MT	Metallothionein
GSSG	Oxidized glutathione

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INTRODUCTION

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INTRODUCTION

Antioxidant is any substance that reduces oxidative damage (damage due to oxygen) such as that caused by free radicals. Free radicals are highly reactive chemicals that attack molecules by apturing electrons and thus modifying chemical structures.

Well- known antioxidant include a number of enzymes and other substances such as vitamin C, vitamin E and beta carotene as well as methionine (Sulfer- containing amino acid) that are capable of counteracting the damaging effects of oxidation.

Antioxidants are also commonly added to food products like vegetable oils and prepared foods to prevent or delay their deterioration from the action of air.

Because vitamin C can donate electrons, it effectively prevents damage caused by free radicals. Its role as an antioxidant is well established through many animal and human studies.

Methionine plays an important role in antioxidant defense mechanism by reacting readily with oxidant to form methionine sulfoxide **(Livine *et al.,* 1999)**.

Lead and cadmiun are included in metal -induced oxidative stress because these metals are commonly found in the environment and have been suggested as being very toxic and carcinogenic agents.

Beneficial role of ascorbic acid and methionine alone or with chelators has been reported earlier against lead toxicity using rat as experimental animal **(Patra *et al.*, 2001 and, Patra and Swarup, 2004).**

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Ascorbic acid is a potent scavenger of superoxide anion radicals and singlet oxygen, and their increased concentration in muscle of rats can contribute to the better protection against Cd toxicity **(Pavlovic *et al* ., 2001)**

Liver and kidney are important organs of metabolism, detoxification, storage and excretion of toxic substances and their metabolites, and especially vulnerable to damage. Liver and kidney are the primary target for toxic effects of Cd and Pb as evidenced by clinical manifestations and biochemical alterations. Cell membranes are also targets for oxidative damage produced by xenobiotics including heavy metals **(Halliwel and Gutteridge, 1990).**

The current study was designed to investigate and to assess the protective effects of vitamin C and DL- methionine on lead and cadmium toxicity , they were tested separately for their toxic effects , where such heavy metals induce abroad range of biochemical dysfunction.

A dose of 300 ppm of Pb or 40mg Cd through drinking water for five weeks have been administered to study the weekly body weight of female rats and its organs as well as some biochemical parameters in blood serum i.e: serum total protein, AST, ALT, glucose, urea, creatinine, total and indirect bilirubin, triglycerides, total Cholesterol, as well as HDL- and LDL- cholesterol.

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REVIEW OF LITERATURE

Antioxidants can be conceptualized in a number of different ways:as insurance against some of the more visible effects of aging , as a weapon in our fight to make our average life expectancy more closely resemble our ultimate lifespan , and as a line of defence against the risk of devolping certain illinesses and diseases . Essentially they are substances that reduce, neutralize, and prevent the damage done to the body by free radicals. Free radicals are simply electrons that are no longer attached to atoms. Instead of circling the nucleus of an atom, free radicals are both free and radical enough to go careening through our cells, inflicting damage as they go.

A process called oxidation creates free radicals and this process happens in the context of normal metabolic processes and our everyday exposure to our environment. In other words, eating, drinking, breathing, and going out in the sun all contribute to the process of oxidation, free radical formation, and the resulting damage that is caused to the cells of our bodies. So minimizing and neutralizing free radicals activity with antioxidants may allow us to live longer and healthier lives, look and feel better, and reduce or eliminate the risk of certain diseases or protect us from the oxidative damage effects of pollutants (especially heavy metals).

Definitions of Antioxidants: -

Antioxidants have been defined "as any substance which delays or inhibits oxidative damage to a target molecule" .In general, an antioxidant in the body may work in one of five ways:

1. Replacing damage ”target molecules”.
2. Keeping formation of reactive species to a minimum.
3. Repairing damage “target molecules”.

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4. Binding metal ions required for formation of highly reactive species (such as $^{\cdot}OH$).
5. Scavenging reactive species either by using enzymes or directly by reaction where by the antioxidant it self would be used up.

An antioxidant is a chemical that prevents the oxidation of other chemicals. In biological systems, the normal processes of oxidation produce highly reactive free radicals. These can readily react with and damage other molecules. The presence of extremely easily oxidisable compounds in the system can "mop up" free radicals before they damage other essential molecules.

The word "antioxidant arises from the chemical definition of "oxidation" and "reduction". A free radical is an "oxidant" "oxidizer" or "oxidizing agent ", a chemical that wants to take an electron away from another chemical and use that electron to stabilize it self. This damages or oxidizes the other chemical. An antioxidant is a "reducer" that sacrifices its electron to the free radical. When a free radical and an antioxidant react, the free radical is reduced. It has gained an electron, and the antioxidant is oxidized. It has lost an electron.

There are two groups of antioxidants, the preventative antioxidants (as vitamin E) and the radical- scavenging antioxidant (as vitamin C).

Cell membranes are targets for oxidative damage produced by heavy metals **(Leonard *et al.*, 2004).** Decomposition of membrane lipids is disastrous for living systems. The protection of cells against damage from oxygen and its metabolites can be accomplished through enzymatic and non-enzymatic means. Antioxidant enzymes such as superoxid dismutase (SOD), catalase (CAT), glutathione peroxidase (GP_X), and the non – enzymatic antioxidant namely ascorbic acid, α-tocopherol, reduced glutathione (GSH) and total sulfhydryl (TSH) scavenge free radicals and lipid peroxides and detoxify them **(Dwivedi *et al.*, 1984).** Several reported have shown that lipid peroxidation is enhanced by

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disturbances such as depletion of cellular antioxidants (**Cine and Moretti , 1995; Pompella *et al.*, 1991**).

Nordenson and **Beckman, (1981)** pointed out that superoxide dismutase and catalase are the two most important radical scavenging enzymes and body's secondary defense against oxygen metabolites produced due to transitional heavy metals.

Eriksson (1982) classified the antioxidants according to their function into: Primary or free radical terminators which act as by terminating the free radical chain in lipid oxidation. Secondary that act by breaking down the hydroperoxides elicited during lipid oxidation into stable end products.

Hamama *et al.*, (1987) stated that antioxidants may regulate liver and serum total protein by stimulating certain endocrine gland for excretion hormone which regulate protein metabolism.

Frei *et al.*, (1989) stated that a number of enzymatic and non-enzymatic antioxidants have been evolved to protect living organisms against free radical-mediated cellular damage.

Krinsky (1992) defined the biological antioxidants as compounds that protect biological systems against the potentially harmful effect of processes or reactions that cause extensive oxidations.

Navasumrit *et al.*, (2000) reported that antioxidants propably exert their effects through their ability to scavenge reactive oxidants.

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Patra *et al.,* (2001) and Ramanthan *et al., (*2002) reported that antioxidant have been found beneficial to mitigate chemical induced oxidative damage.

Mceigot *et al*, (2005) reported that animal cells contain a variety of non – enzymatic small molecular antioxidant that block the effects of ROS. These include glutathione (GSH) and ascorbic acid. The later reduces highly reactive radicals such as hydroxyl, peroxyl and superoxide radicals as well as singlet oxygen and reactive peroxides.

Methionine (sulfur – containing amino acid)

Metionine: essential amino acid, acts against the urinary system infections and against chronic renal insufficiency, limited capacity of the kidneys to eliminate urinary substances, the physiological supplier more important of the methyl and sulphur groups, disorders in the absorption of metionine may lead to mental incapacity, spasms, fetid diarrheas, and premature gray.

Methionine helps the body process and eliminates fat. It contains sulphur , a substance that is required for the production of the bodies most abundant natural antioxidants, glutathione. The body also needs plenty of methionine to produce two other S- containing amino acids, cysteine and thiamine, which help the body eliminate toxins , build strong, healthy tissues, and promote cardiovascular health.

Methionine is a lipotropic, or a chemical substance helps the liver process fats (lipids). It helps prevent the accumulation of fat in the liver and thus ensure normal liver function, which is essential for elimination of toxins from the body. Also it supports liver function by regulating glutathione supplies, gltathione is

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needed to help neutralize toxins in liver. Methionine is needed to make creatine, a nutrient naturally found primarily in muscle tissue that provides the energy of our muscles need to move .

Suzuki and **Cherian , (1989)** exploited the role of glutathione in the interaction with toxic metals., the current administration of methionine , a sulphydryl amino acid and a precursor of glutathione has been shown to increase the efficacy of chelating agents in the treatment of lead **(Tandon *et al* . 1994)** and cadmium intoxication .

Thiamine, a sulfur – containing vitamin has also been used to eliminate heavy metals especially lead **(Swarup** and **Upadhyaya , 1991)** and cadmium **(Gubrelay *et al* .1998).**

Reed and Orrenius, (1997) reported that both amino acids cysteine and methionine reduced lipid peroxidation and restored SOD and catalase activities in liver and kidney . Better protection was recorded in liver with concomitant use of cysteine, which might be due to its easy up take by the hepatocytes for the synthesis of glutathione. **Quig , (1998)** reported that in addition to glutathione synthesis , thiol groups might have helped in chelation of arsenic from the tissue through the synthesis of bile acids thus facilitating removal of arsenic – sulfur complex from liver .

Reed and **Orrenius , (1997)** reported that methionine , a precursor amino acid for glutathione synthesis , protects cells from oxidative damage and plays vital role in detoxification of xenobiotics. The metabolic derivatives of methionine, glutathione, reduced (GSH), is a reference antioxidant component of living organisms.

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Tandon *et al.* (1997) reported that the enhanced efficacy of chelating agents also may be attributed to the ability of methionine to facilitate the cell penetration of chelaling agents by reducing their ionic character.

Livine *et al.* (1999) reported that methionine plays an important role in antioxidant defense mechanism by readily with oxidant to form methionine sulfoxide.

Levine *et al* (2000) studied the role of methionine residues in protein.They concluded that methionine,like cysteine, functions as an antioxidant and as a key component of a system for regulation of cellular metabolism.

Hantson *et al.*, (2003) and **Flora *et al.*, (2004)** reported that dimercaptosuccinic acid , an antidote belonging to the mercapto family has vicinal dithiol moiety for the binding of arsenic and has been found as a potentially useful drug for the treatment of arsenic poisoning

Nandi *et al*, (2005) reported that methionine or ascorbic acid increase the resistance of hepatocytes to oxidation, activate liver enzymes (AST, ALT and lactate dehydrogenase) and lower the reduced glulathione concentration in the liver.

Kokilavani *et al.* (2005) reported that the levels of free radicals produced during the intoxication of arsenic in rats showed a marked increase in the production of reactive oxygen species (ROS) both in liver and kidney of rats fed with arsenic in drinking water. They added that the combinatorial administration of DL-α-lipoic acid and meso2, 3 dimercaptosuccinic acid to arsenic exposed

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rats significantly decreased the outcome of (ROS) thus bring back the change to near normalcy.

Ascorbic acid (Vitamin C)

Vitamin C is an important antioxidant and free radical scavenger. Free radicals are incomplete atoms or molecules that are the natural by-products of everyday metabolic, detoxification, and immune system processes. These unstable compounds attack nearby molecules in an attempt to become complete.

Antioxidants like Vitamin C are the body's natural defense system against free radicals. Antioxidants are molecules that can safely neutralize these harmful substances, helping to promote cell health.

Ascorbic acid is an important nutrient necessary for human life. Vitamin C corrects the world's oldest known nutritional deficiency and has been heavily researched for its role in a long list of functions in the body. Studies have shown that Vitamin C is beneficial in maintaining general health and may have application in the treatment and prevention of a variety of conditions.

Elwood *et al.*, (1970) found that high doses of Vit.C reduced cholesterol concentration in plasma and tissues of experimental animals .

Nambisan and Kurup (1974) found a statistically significant decrease in plasma triglycerides level due to feeding rats with vitamin C supplement.

Holloway and Rivers (1978) reported that in laboratory animals, vitamin C stimulated the rate- limiting reaction in the conversion of cholesterol to bile acids.

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Bratton *et al.*, (1981) reported that ascorbic acid has earlier been reported as a possible chelator of lead with similar potency as that of EDTA.

Schvartsman (1983) Showed that Vitamin C may exert its protection against environmental pollutants through stimulation of liver detoxifying enzymes. For this reason vitamin C has been suggested as a treatment for certain acute intoxications.

Poulter *et al.*, (1984) pointed that current evidence suggests that the major benefit of ascorbic acid with regard to cancer may be in reducing the risk of developing cancer, rather than in therapy.

Flora and Tandon (1986) reported significant ameliorative efficacy of ascorbic acid and methionine in experimental lead toxicity in rats when given simultaneously as compared to post exposure treatments.

Liberman and Bruning (1990) reported that an increase in Vit.C is related to higher levels of high density lipoproteins (HDL- cholesterol) which appears to protect from heart disease.

Barja *et al.*, (1996) suggested that vitamins E and C at medium concentrations were needed for optimal protection against lipid peroxidation.

JayachandRan *et al.*, (1996) found that total lipids were decreased markedly after ascorbic acid supplementation (20 mg / kg bw/day) in aged rats.

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Barroso *et al.*, (1997) and Ramanathan *et al.*, (2005) reported that administration of Vit.C along with arsenic significantly reduced the extent of apoptosis. This antioxidant vitamin directly inhibits free radical mediated apoptosis by directly eliminating them. A part from the free radical scavenging property antioxidant are known to regulate the expression of number of genes and signal regulatory pathways and there by may prevent the incidence of cell death **(Allen** and **Tresini,2000).**

Stoyanovsky *et al.*, (1998) reported that Vit. C is water – soluble hydrophilic antioxidant that protects cells from oxidative stress by scavenging free radicals.

Pavlovic *et al* ., (2001) reported that ascorbic acid is potent scavenger of superoxide anion radicals and singlet oxygen , and their increased concentration in muscle of rats can contribute to the better protection against Cd toxicity . They added that the increased accumulation of Cd increases production of malonedialdhyde (MA) which consequently inhibits the enzyme L-gulconolactone oxidase necessary for the synthesis of ascorbic acid, (**Hudecova** and **Ginter, 1992**).

Patra *et al.* (2001), and **Patra** and **Swarup, (2004)** reported that the beneficial role of ascorbic acid and methionine alone or with chelators has been reported earlier against lead toxicity using rat as experimental animal.

Hashim and Weshahy (2002) reported a significant decrease in total protein and total bilirubin in blood of female albino rats treated orally with vitamin C (10 mg/Kg bw) compared with control. They also pointed out to the protective role of antioxidant (ascorbic acid) against hemoglobin degradation

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and consequently decreased the level of serum total bilirubin in rats kept on ascorbic acid supplemented diet.

Acharya *et al.* (2003) showed that depletion of endogenous ascorbic acid level in tests suggests its constant use in scavenging the reactive oxygen species (ROS), there by protecting the organ from potential injury.

Ozdil *et al.* (2004). studied the protective effects of ascorbic acid on ethanol – induced liver damage of rats. They reported that as a result of ethanol, enzymes such as AST, ALT and LDH were released in to the blood. The increase in the serum activities of theses enzymes were directly proportional to the degree of cellular damage. In their study, these values (AST, ALT) decreased by administration of Vit. C. The decrease in those increasing enzyme levels shows that Vit.C prevented damage in the liver.

Sadek (2004) showed a remarkable reduction in MDA concentration in liver homogenate of rats received Vit.C at dose of 400mg / day compared to control animals. He suggested that Vit.C or its metabolites combine with MDA to from soluble complex, which is eliminated in urine as reported by **Flora and Tandon (1986).**

Ramanathan *et al.* (2005) observed that ascorbate selectively altered the extent of DNA damage. They added that Vit.C has proved evident as efficient drug against arsenic mediated toxicity and has pointed the way towards the therapeutic implementation of this drug to human exposed to arsenic. They reported that ascorbic acid given at 200 mg/k body weight in acute arsenic toxicity protected tissues from lipid peroxidation and associated changes in antioxidant enzymes.

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Nandi *et al.*, (2005) reported that arsenic exposure resulted in significant increase in endogenous lipid peroxidation and decreased catalase activities in erythrocytes with non – significant changes in superoxide dismutase activities. The simultaneous oral treatment with ascorbic acid during exposure did not result in significant increase in erythrocyte malondialdhyde (MA). However, groups treated with cysteine , methionine and thiamine (sulphur- containing) have significantly higher lipid peroxidase (LPO) than control groups . Cysteine and ascorbic acid treated animals had catalase activity higher than positive control.

Chang *et al.*, (2007) reported that arsenite reduced GSH levels on mouse tests , and this oxidative stress- induced change in reversed by ascorbic acid .

THE TOXICITY OF HEAVY METALS:

The toxicity of any metal, as of any substance, solid, fluid or gaseous, depends upon a great many factors: the nature of its compounds , its mode of entry into or contact with the body or tissue, or skin surface, its concentration (depending on the metal dose and exposure duration) in the atmosphere and the susceptibility of the individual exposed to its effect .

Cadmium :-

Cadmium is a very toxic heavy metal and an important environmental pollutant, which present in the soil, water, air, food, and in cigarette smoke. Cd causes poisoning in various tissues of humans and animals (**Swiergosz *etal.*, 1998**). After the intake and resorption Cd enters the blood where it binds to the erythrocytes membranes and plasma albumin (**Bauman *et.al.* 1993**). In the blood and tissues, Cd stimulates the formation of metallothionines (**Simpkins**

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***et.al.* 1998**) and ROS thus causing oxidative damage in erythrocytes and in various tissues, which results in a loss of membrane functions **(Sarkar *et.al.*, 1995**).

The International Agency for Research on Cancer (IARC) has classified Cd as a group I carcinogen in humans (**1993**). It is present in everyday life and currently it is being implicated as a toxic and carcinogenic agent. It causes lesions to several organs of humans and animals. Major routes of Cd intake into the organism are respiratory and gastrointestinal track, **(Gupta *et.al.*, 1990).**

Over time, dietary intakes of Cd greater than the provisional tolerable weekly intake (7 µg/kg body weight) established by World Health Organization (**WHO, 1989**) can increase the body burden of this toxic elements to the point of causing renal proximal tubular dysfunction.

Food is the major source of Cd exposure for non- smokers, The daily intake of Cd is estimated to be approximately 30µg, with the largest contribution from grain cereal products, potatoes and other vegetables. Exposures through drinking water or ambient air typically are very low (**ATSDR. 1999**).

Cd is a non – physiological heavy metal, The association of Cd with pulmonary, prostatic and testicular cancer may be related to the ability of Cd to induce oxidative stress, which could in turn cause oxidative damage to DNA. **Ikediob *et.al.*, (2004)** found that the increase in ROS production is an indication of oxidative stress, which is known to impact on the performance of oxidative enzymes and metabolites in the cell.

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Elez, *et.al.*, (2001) reported that the major site of the initial cadmium accumulation and toxicity in the body is the liver .

Chan and Cherian (1993) reported that, when Cd enters the body it reaches the liver within the first 6 h and binds to metallothionein , which is protein with low molecular weight (6000-10.000 Da) , and which is rich in cystein . The Cd – metallothionein complex generated in the liver was reported to be mainly distributed to the kidney and other tissues and hence it causes damage in theses tissues. Maetallothionein, play roles in Cd toxicity , transportation and detoxification .

Pavlovic *et.al,* (2001) indicated that chronic Cd administration induced elevated activities of total SOD in muscle of rats. After being introduced into the organs Cd enters the blood and tissues and induces an increased production of reactive species, including. superoxide anion radicals and hydrogen peroxide . As a biological response it is reasonable to expect an increased activity of SOD in muscle, since in physiological conditions SOD is an important intracellular antioxidant, because it eliminates superoxide anion radicals when it is formed in excess.

Cd also depletes glutathione and protein –bound sulphydryl groups, resulting in enhanced production of reactive oxygen species (ROS) result in increased lipid peroxidation, enhanced excretion of urinary lipid metabolites, modulation of intracellular oxidized states, DNA damage, membrane damage, altered gene expression and apoptosis (**Stohs *et.al.*, 2000).**

Yalin *et al.*,(2006) reported that the effect of Cd uptake in cells has been shown to be drastic , normally leading to cell death depending on the metal dose and exposure duration , Acute and chronic Cd exposure also affects antioxidant

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enzyme system in different ways . In their study, the acute effect of Cd was found to have a significant effect on lipid peroxidation and oxidative stress.

Effect of Cadmium on biochemical constituents of serum

Brzoska *et al.*, (2003) concluded that the morphological observation, together with the functional tests show that Cd administrations lead to liver and kidney injury passing a serious risk of health .

Bonnell (1955, 1965) has reviewed all the evidence incriminating Cd as a nephrotoxic agent and has concluded that kidney damage does occur in Cd poisoning. He added that the evidence of damage to the kidneys has been more on the excretion of a special from of protein in urine.

Webb and Samarawckrama, (1981) reported that Cd causes a severe decrease in the activity of thymidine kinase, a rate – limiting enzyme for DNA biosynthesis in embryos.

Shukla *et al*, (1987) reported that their studies have shown that Cd can inhibit the activity of most enzymes of antioxidant defense system.

Vig and Nath , (1991) and **Paier *et al.* (1993)** reported that Cd can interact with sulfhydryl or carboxyl groups of several enzymes and, thus. interfere with the formation of enzyme substrate complexes.

Rana and Boora (1992) indicated that Cd alters GSH levels, Cd administrated orally for 30 days was found to increase GSH levels in rats. GSH is known to protect cells against oxidative stress. They showed that Cd toxicity causes oxidative stress by challenging the thiol status of cells.

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Jarup ***et al.*****, (2000); and Jarup , (2002)** observed the damaging effect of Cd on kidney.

Pavlovic ***et al.*****, (2001).** observed a significant accumulation of Cd in muscles of rats treated with Cd. They concluded that this is in accordance with the fact that Cd in the organism is not metabolized and mostly accumulates in cells and tissues. Most of Cd accumulates in the cytosol where it induces many structural and metabolic changes.

Swiergosz ***et al.*****, (1998)** reported that Cd decreases protein synthesis and inactivate enzymes by binding to their sulfhydryl groups. These facts can explain a decreased of total protein level in muscles of rats treated with Cd.

Klaassen and Liu, (1997) and Gong and Hart, (1997) showed that metals (particularly Cd) are stored as Cd –MT (metallothionine) complex in the liver. This Cd – MT is transferred from the liver to the kidneys over time, and then filtered and reabsorbed by the renal proximal tubules. Cd – MT is metabolized in lysosomes to liberate Cd ions. These liberated Cd ions again bind to preexisting or newly made MT. If the MT synthesis cannot keep up with the demand and the non – MT bound Cd overwhelms other defense systems, Cd toxicity resulted (ensues).

Yamano ***et al.*****, (1999) and Yiin** ***et al.*** **(1999)** reported that having been absorbed from the alimentary tract, Cd forms durable combinations with the protein thionein, forming metallothioneins which play an important role in further metabolism of this metal. Kidneys (mainly renal cortex) and liver are considered to be the most susceptible organs in the case of exposure to Cd.

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Simpkins, (2000) concluded that Cd remains in the lungs and induces a cysteine rich protein called metallothionine (MT). Which are known to protect cells from oxidative stress, since MT is comparatively cysteine - rich (20-30% of protein is Cys), and metals have a high affinity for thiols, MT is known to sequester metals.

Pavlovic *et al.*, (2001) presumed that Cd enters the mitochondria and inhibits the activities of many enzymes by binding to their sulfhydryl group or by inhibiting the protein synthesis.

Ivanov *et al.*, (2003) pointed out that the effect of Cd on the protein synthesis *in vivo* depends on intoxication duration and, probably, on dose of this metal. They added that according to the data of in vitro study, Cd in low concentrations can activate both the rate and the level of translation but in high concentrations it inhibits those parameters.

Cheesbrough (1992) observed that, a rise in total bilirubin in serum of Cd-treated rats suggest liver cell damage in which, usually, there is an increase in both total bilirubin and conjugated bilirubin. Another possible reason may be a metabolic disturbance in liver involving defective conjugation and or excretion of bilirubin. The bilirubin route of elimination is perhaps most important contributing source to the excretion of the animal metabolite since liver encounters nutrients, environmental toxicant and waste products, so it extracts the environmental toxicants and waste products to prevent their circulation to other parts of the body.

Sauer *et al.* (1997) and Blasco and Puppo (1999) also observed an increase of the activity of ALT and AST enzymes as a result of Cd activity.

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Sarkar *et al.,* **(1998)** found that the increase of total bilirubin concentration in plasma after intoxication with Cd correlates with the oxidative damage of other organs resulting from oxidative stress.

Novelli ***et al.*****, (1998)** pointed out that another sensitive indicator of liver cell damage is the concentration of total bilirubin in serum (plasma). They reported that a significant increase of total bilirubin concentration in rats exposed to Cd.

As a result of Cd activity one notices renal tubule damage and then glomerular filtration impairment **Shibutani** ***et al.*****, (2001)**. This may account for the increase of urea and creatinine concentration in the animals receiving $CdCl_2$ **Kowalczyk** ***et al.*****,(2003)**.

Kowalczyk ***et al.*****, (2003)** reported that administering $CdCl_2$ to rats resulted in significant decrease of hemoglobin concentration in blood in relation to control group. They added that AST and ALT activities, concentrations of bilirubin, urea and creatinine in blood serum were increased as a result of Cd administration. They stated that the damaging effect of Cd on liver is manifested by an increase of aspartate aminotransferase (AST) and of the most specific marker of liver cell damage – alanine transferase (ALT). They added that cadmium hepatocity is probably effected in two ways, on the one hand by the occurrence of inflammatory state, on the other hand – by direct toxic action of Cd on liver cells.

Brzoska ***et al.*****, (2003)** reported that Cd exposure affected some biochemical markers of kidney function. Exposure to Cd alone increased the serum urea concentration (by 16%), but had no effect on the total protein

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concentration in serum. Moreover, it increased the activity of ALT and AST when measured versus control.

Yiin and Shei (1998) indicated that when Cd concentration, exposure time, and temperature of incubation (*in vitro*) were increased, the production of lipid peroxidation was also elevated.

Ercal *et al.*, (2001) reported that lipid peroxidation has been observed in Cd toxicity. They showed that disturbances in GSH and MT levels may allow free radicals to be "free" such that HO ˙. and $O2^{-}$ radicals can attack double bonds in membrane lipids and result in an increase in lipid peroxidation.

Lead:-

Lead is a poisonous metal that can damage nervous connections (especially in young children) and cause blood and brain disorders. The concern about lead's role in mental retardation in children has brought about widespread reduction in its use (lead exposure has been linked to schizophrenia). Lead is considered to be particularly harmful for women's ability to reproduce. For that reason many universities do not hand out lead- containing samples to women for instructional laboratory analysis.

Prolonged exposure to lead causes an accumulation of the element in the body. That can result in a variety of mental and physical problems: reading and learning disabilities, speech and language handicaps, mental retardation, kidney disease and heart disease.

Much of the lead taken into the body is incorporated into bone where it constantly interchanges with other tissues.**(Hu *et.al.*,1998)** Recent studies suggest that accumulated lead exposure is related to several chronic disorders of aging including hypertension and cognitive decline **(Vig and Hu , 2000),**

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disorders that have been associated with oxidative stress. **(Romero –Alvira and Roche, 1996; Mecocci *et al.,* 2004).**

Symptoms of lead poisoning

- no desire to eat food
- loss of recently acquired skills
- drowsiness
- irritability
- headache
- lack of energy
- constipation
- stomach cramps
- trouble sleeping

Lacranjan *et al.,* (1975) and Bauchinger *et al.,* (1976) reported that lead – induced hypofertlilty particularly, among the workers of lead factories, it has been thought to be due to the direct toxic effects on male gonads.

Approximately 90% of the total body lead is contained with in bones **(Friberg *et al.*, 1979)**. Blood accounts for 4 % and the remain lead resides mainly in the liver and kidneys **(Boeckx, 1986)**. The liver and kidneys are also known to play a major role in the elimination of lead **(Goyer and Cherian, 1979)**, and hence account for the toxic actions **(Lockiteh, 1993)**.

Lead is one of the most harmful heavy metals, able to induce renal, hepatic and testicular injury. Studies have shown that Pb has the ability to produce ROS, resulting in lipid peroxidation and DNA damage. Lead can be concentrated in the nucleus and can perturb cell proliferation and DNA synthesis *in vivo* **(Pepenoe** and **Schamaeler, 1979, Gennart *et al.*, 1980)**. These

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alterations may occur via indirect mechanism including disturbance of enzyme functions important in DNA repair.

Stefan and **Benow (1981)** reported that like all other heavy metals, Pb is known to induce oxidative stress in animals indicated by a sharp rise in lipid peroxidation potential (LPP).

Gibbs *et al.*,(1985) have reported that gestational administration of Pb decrease hemoglobin synthesis in rats due to the inhibition of enzymatic activities implicated in the heme group synthesis, particularly aminolevulinic acid dehydratase and ferrochelatase .

Heffner and **Repine, (1989)** reported that reactive oxygen radicals (ROS) resulted from lead exposure to mice are detrimental to tests and therefore, are regularly being scavenged by a variety of endogenous antioxidants and quenchers including vitamins, and tripeptides .

Chaurasia and **Kar (1997)** reported that lead is known to produce oxidative damage on the liver tissues by enhancing peroxidation of membrane lipids , and a deleterious process slowly carried out by free radicals **(Halliwell and Gutteridge, 1990)**.

Gurer *et al.*, (1999) and Somashekaraiah *et al* ., (1992) reported that lead is known to produce oxidative damage in the liver by enhancing lipid peroxidase (LPO) .

Effect of lead on biochemical constituents of serum

Several studies investigated the toxic effect of Pb on membrane components and found a direct correlation between these effects and Pb induced oxidative damage.

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Davis and Berndt, (1994) reported that changes of serum urea and creatinine have been used as important indices for the evaluation of the effect of chemicals on kidney. The presence of increasing urea and creatinine concentration in the blood suggest the inability of kidney to excrete these products, which further suggest a decrease in glomerular filtration rate (GFR).

Lewis and Wills, (1962) have suggested that peroxide formation may lead to oxidative destruction of thiol groups of amino acids and proteins. Reports on lead – induced oxidative stress dates back to 1965 **(willis, 1965).** Lead can cause derangement of several hepatic biochemical pathways and energy metabolism **(Taki *et al.*, 1985)**.

Needleman and Bellinger, (1991) reported that lead also can interact with some amino acids (Cys, Asp, Glu, Tyr) of enzymes, these might alter protein synthesis and produced a decrease in total protein amount.

Goering (1993) reported that lead binds to plasmatic proteins and is conducted to liver , where it causes alterations of high number of enzymes . It can also perturb proteins biosynthesis in hepatocytes , including the structural proteins .

Mobilization of hepatic glycogen stores and altered activities of the gluconeogenic enzymes pyruvate carboxylase, phosphoenolpyruvate carboxykinase , fructose -1.6-diphosphatase , glucose -6- phosphatase , and glucose -6- phosphate dehydrogenase following Pb exposure , have been reported by **Hacker, *et al.,* (1990)**.

Calabrese and Baldwin , (1992) concluded that one of the described effects of Pb exposure in experimental animals is glycosuria, because metal delays the release of insulin from the pancreas and suppresses its activity. In

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addition, this toxicant increases cellular cAMP content, which results in increased, glucose levels. Their studies have shown that gluconeogenesis is inhibited in rats after chronic lead intoxication, although it is most likely to be associated with Pb biotransformation and liver cell proliferation in adult Wister rats.

Corpas *et al.*, (2002) concluded that Pb alters glucose metabolism where blood glucose levels increased and in accordance, hepatic glycogen levels decreased by 48 – 61 % in intoxicated pups. They added that since they have observed high glucose levels, the use of lipids to generate glucose was possibly not necessary and, consequently, differences in hepatic lipids could be attributed entirely to alterations in the biosynthesis due to Pb administration.

Liss *et al.*, (1985) reported that aminotransferase (ALT and AST) is an important class of enzymes linking carbohydrate and amino acid metabolism. These enzymes are regarded as markers of liver injury, since liver is the major site of metabolism.

Halliwell and Gutteridge (1990) reported that redox disturbances are known to negatively impact body system through generation of reaction oxygen species (ROS), which modify proteins, lipid and DNA. They added that liver being one of the targets for lead accumulation, has witnessed the toxic insult lead by way of decrease in the activities of transaminase.

Studies have reported that Pb has an effect on *de novo* lipid synthesis in animals, according to the range of dose (**Knowles and Donaldson, 1990**) and it causes lipid abnormalities by inhibition of hepatic lipase activity in rats with low dose of metal **(Skoznynska *et al.*, 1993).**

Skoznynska *et al.*, (1993) have reported high plasma triglyceride levels in intoxicated rats, suggesting that they were due to the inhibition of lipase lipoprotein.

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Lawton and Donaldson ,(1991) suggested that arechidonic acid augmentation might be responsible for the enhanced lipid peroxidation in cellular membrane.

Shafiq – ur – Rehman and Abdulla, (1993) observed that lead is shown to strongly bind to phosphatidylcholine membranes *in vitro* thereby decreasing the levels of phosphatidyl choline .

Yiin and Lin (1995) reported that when Pb was incubated with various polyunsaturated fatty acids a marked enhancement in MDA concentration was observed. MDA concentration was found to increase as the number of double bonds of fatty acids increased.

Othman and Elmissiry (1998) administered intramuscularly a single dose of Pb (100μ mole / kg b.wt) for male albino rats. After 3 and 24 hours, Pb treatment resulted in significant increase, ALT and AST, total proteins and cholesterol in serum. The total triglyceride in serum was decreased after 24 hours of intoxication. Lead treatment also produced significant elevation of lipid peroxidationin liver and kidney.

Sivaprasad *et al.*, (2004) reported that the overall inhibitory effects of Pb on various enzymes would probably result in impaired antioxidant defense by cells and would render them more prone to oxidant attack. They showed that Pb administration caused a significant decrease in the activities of ALT (- 38%) and AST (- 42%) also caused significant increase in LPO and decrease in the antioxidant enzymes CAT (- 45%), SOD (- 46%). Further more, Pb administration caused decreases in GSH (- 43%) and the glutathione metabolizing enzymes GR (-59%), G6PD (-27%) and GST (- 42%).

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How: Heavy metals – induce oxidative stress?

Ercal *et al.*, (2001) proposed mechanisms for Pb – induced oxidative stress in four groups: 1) Direct effect of lead on cell membranes. 2) Lead hemoglobin interaction. 3) δ- aminolevulinic acid (δ-ALA) – induced generation of ROS, and 4) Effect of lead on the antioxidant defense systems of cells.

However, proposed mechanisms for Cd – induced oxidative stress can be studied in three groups as reported also by **Ercal *et al.*, (2001) :**

1) Adverse effects of Cd on cellular defense systems and thiol status,

2) Enhancement of lipid peroxidation by Cd , and 3) Deleterious effect of Cd on cellular enzymes .

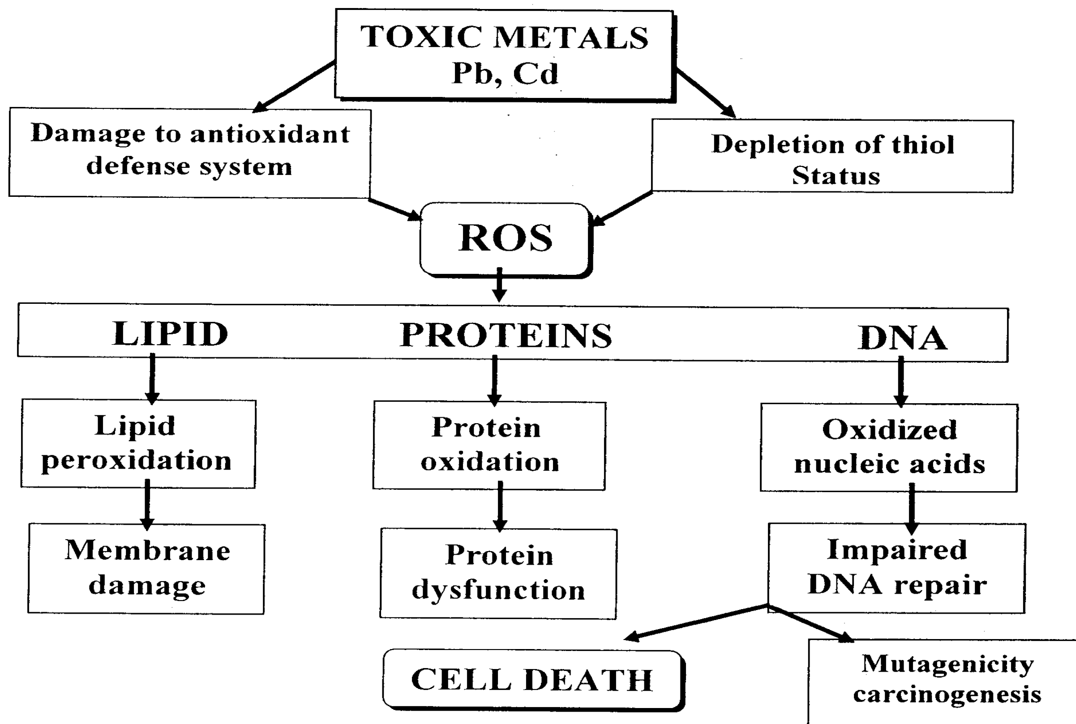

Figure (i) show the possible mechanisms for metal – induced oxidative stress provided by **Ercal *et al.*, (2001).**

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Mylroie *et al.*, (1984) reported that antioxidant enzymes , which remove peroxide , and superoxide radicals including glutathione peroxide (GPX) , catalase (CAT) and superoxide mutase (SOD) , are potential targets for lead . Lead is known to inhibit heme synthesis, and since CAT is a heme – containing enzyme, it cause CAT activity to decrease.

Comporti (1985) emphasized the possible relationship between lipid peroxidation (LPO) and cellular damage in hepatic tissues under various pathological conditions.

Manca *et al.*, (1991) found that both cations Cd and Pb alter the biosynthesis of nucleic acids

Halliwell , (1994) reported that lipid peroxidation is a basic cellular deteriorating process induced by oxidative stress and occurs readily in the tissues rich in high oxidizable polyunsaturated fally acids .

Othman and El-Missiry , (1998) reported that cells have developed various antioxidant defense systems against free radical attacks . GSH, 8-glutamyl – cysteinyl – glycine , plays a major role in protecting cells against oxidative stress . GSH has carboxylic acid groups, an amino group, asulfhydryl group and two peptide linkages as sites for the reaction of metals. Its functional group, - SH, plays an important role in metal binding. Glutathione reductase (GR) reduces glutathione disulfide (GSSG) to GSH, thereby supporting the antioxidant defence system. GR has a disulfide bond in its active site. But lead interferes with the disulfide bond and inhibits the enzyme. This inhibition prevents the reduction of GSSG, making cells more susceptible to oxidative damage. They add that the antioxidant capacity of hepatic and renal cells of Pb treatments in terms of the activities of superoxide dismutase , glutathione

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reductase , and glutathione content was diminished . They added that Pb may exert its toxic effect via peroxidative damage to renal and hepatic membranes after 24 hours.

Gurer and Ercal (2000) studied the direct effect of lead on cell membranes; they reported that lead exposure probably may further increase the susceptibility of membranes by altering their integrity via causing deterioration of their components.

Sivaprasad *et al.*, (2004) concluded that the impaired antioxidant balance can partially responsible for the toxic effects of lead. Restoration of the cells antioxidant capacity appears to provide a partial remedy against Pb – induced oxidative stress.

Bertin and Averbeck (2006) proposed the general scheme of biological consequence of cadmium intoxication in cells (Fig. ii). Cadmium interferes with various important mechanisms such as gene expression, cell cycle, differentiation and proliferation. Cadmium gives rise to oxidative damage affecting DNA, proteins and membrane lipids. The induction of oxidative damage is associated with mitochondrial dysfunction, deregulation of intracellular antioxidants and apoptosis. Oxidative damage to DNA leads to mutations and induction of cancer. The inhibition of some DNA repair pathways contributes to the rise in mutations and cancer.

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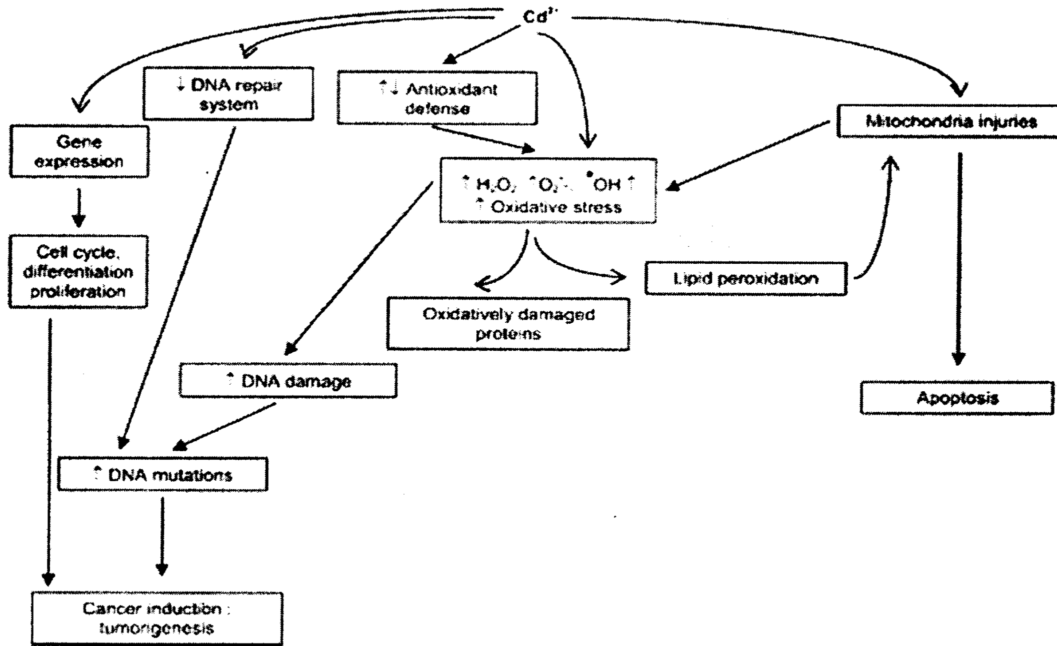

Figure (ii) The General scheme of biological consequences of cadmium intoxication in cells. Provided by **Bertin and Averbeck (2006)**

Effect of heavy metals and antioxidant on rat body weight

Grany *et al.*, (1980) found a reduction on body weight with Pb dose of 250 mg / L .

Hayahi (1983) found that a Pb dose of 200mg / L did not alter body weight of rats .

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Corpas *et al.*, (2002) reported that Pb intoxication had no effect on weight gain or on water consumption in rat mothers during 3 weeks of gestation.

Nandi *et al.*, (2005) studied the effect of cysteine, methionine, ascorbic acid and thiamine on arsenic – induced oxidative stress and biochemical alteration in rats. Rats were given o ppm (group I healthy controls) or 10ppm arsenic in drinking water for a period of 12 weeks. They reported that there was a steady increase in body weight of rats of all the groups. However, the growth was comparatively poor in arsenic exposed untreated rats as evidenced by lower mean body weight from 10 weeks onwards as compared to respective value in control group.

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MATERIALS AND METHODS

Experimental design:

Female albino rats of ***winstar strain*** (age of 6 weeks) weighing approximately 136 ±2.29 g were obtained from the Animals House Unit, Institute of Food Technology, Agriculture Research Centre. A total number of 49 rats were divided into seven groups and were housed in spacious and well aerated cages under hygienic condition for one week.

Sawdust was used as the bedding, which was changed during cleaning of cages on every alternate day. The animals were provided with laboratory animal feed (Diet: casein 20 %, fat as corn oil 10%, vitamins 1%, salt mixture 4% , cellulose 5 % and starch 60 %) obtained from the Feed Unit of the Institute , approximately of 15 g /rat daily . The left over feed on the next day was discarded and fresh feed was provided to them. Drinking water was supplied ad-libitum.

After one week of acclimization period, they randomly assigned into seven groups. Administration of heavy metals in drinking water i.e Pb (groups 2,3and 4) or Cd (groups 5, 6 and7) began from the second week. However, antioxidant administration in diet started from the third week i.e methionine (groups 3 and 6) and ascorbic acid (groups 4 and 7).

The experimental seven groups were as follow:

Group I (n=7) : Rats received diet alone (basal), served as control, the drinking water was administered *ad libitum* as distilled water (control).

Group II : Rats received diet alone, the administered drinking water was as lead acetate solution (Merck) prepared

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in distilled water, and replaced daily to minimize precipitation (Pb: 300mg /L).

Group III : Rats received Methionine – diet (6.5g/kg diet) and drinking water(lead acetate solution, Pb:300mg / L)

Group IV : Rats received Ascorbic acid –diet (250mg /kg diet) and drinking water (lead acetate solution, Pb: 300mg / L).

Group V : Rats received diet alone, the administered drinking water was as cadmium chloride (Merck) prepared in distilled water (Cd: 40mg / L $CdCl_2 .5H_2O$ in drinking water).

Group VI : Rats received Methionine –diet (6.5/kg diet) and drinking water (Cd: 40mg / L CdCl2. 5H2O in drinking water).

Group VII : Rats received Ascorbic acid – diet (250mg/kg diet) and drinking water (Cd: 40mg / L $CdCl_2 .5H_2O$ in drinking water).

The weekly body weight of the individual rat was measured during the entire period of experiment. On the completion of 35 days of experimental period, animals in different groups were scarified under light ether anesthesia at the end of experiment, liver, kidneys, lung, spleen and heart were excised immediately and weighed.

Blood samples were collected, and then were centrifuged at 3000 rpm for 30 minutes after collection. The obtained serum was transferred into clean – capped vials and stored at 25 – 20 °C. until the required analytical procedures were performed.

Biochemical Studies:

All reagents needed for determinations of several biochemical parameters are individually packaged in a commercial kits supplied from Diamond Diagnostics PIG, El-Gomhoria Company for Drugs, Egypt.

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1- Serum total protein

Serum total protein was determined according to the colometric method provided by **Henry (1964).** The commercial kit needed for serum total proteins contained the following:

Components:

Reagent(1): Protein standard (6.0 g/dl).
Reagent(2): Biuret reagent.
NaoH (0.2N), K-Na –Tartarate (18m mol /L),
K-iodide (12 m mol/L) and Cupric sulphate (6.0 m mol/L)

Procedure:

For tubes labeled as blank , standard and sample , 1 ml of (Reagent 2) was added in all tubes and 20μl of standard solution (Reagent 1) were added in standard tube , and 20 μl of serum were added in sample tube . All tubes were mixed and incubated for 5 minutes at 20-25 °C.
The absorbance was measured at wave length 546 nm for sample (A_{sample}) and standard ($A_{standard}$) against reagent blank.

Calculation:

Concentration of total protein (g/dl) was measured using the following equation:

$$\text{total protein conc.} = \frac{\text{A sample}}{\text{A standard}} \times 6$$

Where 6: conc. of standard (g/dl).

2- Aspartate aminotransferase(AST)

The colorimetric method for AST determination was carried out according to the method described by **Reitman and Frankel (1957)** based on the following equation:

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L- glutamate + oxalacetate $\xrightarrow{AST}$ α-ketoglutarate + L-aspartate

Oxalacetate formed is measured by monitoring the concentration of oxalacetate hydrazone formed with 2,4-dinitrophenylhydrazine .

Reagents:

Reagent 1: (Buffer substrate)	Phosphate buffer pH 7.2; L-asparate 80 m mol / l and α-ketoglutarate 4.0 m mol / l.
Reagent 2: (color reagent)	2,4- dinitrophenylhydrazine 4.0 m mol/l.

Procedure:

0.5ml of reagent (1) was added in tubes labeled as sample and blank, then 100 μl of sample (serum) were added in sample tube, and 100 μl of distilled water were added in blank tube. Mix, incubate for exactly 30 minutes at 37 °C then, for both tubes 0.5 ml of reagent (2) was added. Mix, incubate for exactly 20 minutes at 20-25 °C then add 5.0 ml NaOH (0.4mol / L). Mix and read the absorbance of sample (A_{sample}) at wave length 546 nm after 5 minutes against reagent blank.

Calculation:

Obtain the activity of AST in the serum from the following table:

Absorbance	U/L	Absorbance	U/L
0.020	7	0.100	36
0.030	10	0.110	41
0.040	13	0.120	47
0.050	16	0.130	52
0.060	19	0.140	59
0.070	23	0.150	67
0.080	27	0.160	76
0.090	31	0.170	89

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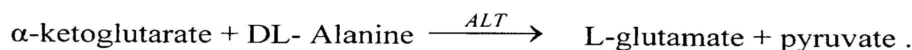
3- *Alanine aminotransferase (ALT)*

All determination was carried out as described by **Reitman and Frankel (1957)** based on the following equation:



$$\alpha\text{-ketoglutarate} + \text{DL- Alanine} \xrightarrow{ALT} \text{L-glutamate} + \text{pyruvate}$$

Pyruvate formed is measured by monitoring the concentration of pyruvate hydrazone formed with 2,4dinitrophenylhydrazine.

Reagents:

Reagent1: (Buffer substrate)	Phosphate buffer pH 7.2; L-asparate 80 m mol / l and α-ketoglutarate 4.0 m mol / l.
Reagent 2 : (color reagent)	2,4- dinitrophenylhydrazine 4.0 m mol/l.

Procedure:

0.5 ml of reagent(1) was added in tubes labeled as sample and blank , then 100 µl of serum (sample) were added in sample tube , and 100 µl of distilled water were added in blank tube . Mix, incubate for exactly 30 minutes at 37 °C then, for both tubes 0.5 ml of reagent (2) was added. Mix, incubate for exactly 20 minutes at 20-25 °C then added 5 ml NaOH (0.4mol/L). Mix and read the absorbance at 546 nm of sample (A sample) after 5 minutes against reagent blank.

Calculation:

Obtain the activity of ALT in the serum from the following table:

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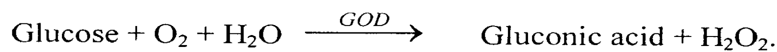
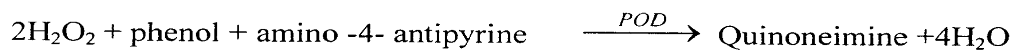
Absorbance	U/L	Absorbance	U/L
0.025	4	0.270	48
0.050	8	0.300	52
0.075	12	0.325	57
0.100	17	0.350	62
0.125	21	0.375	67
0.150	25	0.400	72
0.175	29	0.425	77
0.200	34	0.450	83
0.225	39	0.475	88
0.250	43	0.500	94

4- *Glucose*

Glucose in serum was determined using the enzymatic colorimetric method as described by **Trinder (1969).** Glucose presenting the sample is determined according to the following equation:



$$\text{Glucose} + O_2 + H_2O \xrightarrow{GOD} \text{Gluconic acid} + H_2O_2.$$



$$2H_2O_2 + \text{phenol} + \text{amino -4- antipyrine} \xrightarrow{POD} \text{Quinoneimine} + 4H_2O$$

Reagents:

Reagent(1): Glucose standard (100mg/dL).
Reagent(2): Phosphate buffer pH 7.2 and phenol (10.0 m mol/L).
Reagent(3): Amino - 4 - antipyrine (0.70 mmol /L); Glucose oxidase
(20U /ml) and peroxidase (1.0 U /ml).

Procedure:

Working solution: add the contents of one bottle of reagent (3) to a bottle of reagent (2), and mix well until completely dissolved. Do not shake. The working solution is ready to use after 10 minutes.

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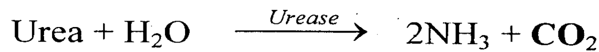
For tubes labeled as blank , standard and sample , 1 ml of working solution was added in all tubes . 10 µl of standard (reagent 1) were added to standard tube, and 10 µl of serum (sample) were added in sample tube. Mix and incubate for 10 minutes at 37 °C. Measure the absorbance at 500 nm of sample (A sample) and standard (A standard) against reagent blank.

Calculation:

$$\text{Glucose concentration mg/dL} = \frac{\text{A sample}}{\text{A standard}} \times 100$$

5- *Urea:*

Enzymatic determination of urea was carried out according to the following reaction as reported by **Patton and Crouch (1977)**:



$$\text{Urea} + H_2O \xrightarrow{Urease} 2NH_3 + CO_2$$

In alkaline medium, the ammonium ions react with the salicylate and hypochlorite to form a green colored indophenol.

Reagents:

Reagent(1): Urea standard (50mg/dL).
Reagent(2): Enzyme reagent (Urease >5000 U/L)
Reagent(3): Buffer reagent: phosphate buffer pH 8.0; sodium salicylate (52 mmol/L); sodium nitroprusside (2.9 mmol/L), and EDTA (2.0 mmol/L).
Reagent(4): Alkaline reagent: sodium hydroxide (80 mmol/L), and sodium hypochlorite (4.0 mmol/L).

Procedure:

For tubes labeled as blank , standard and sample , 1 ml of reagent **(3)** (buffer) was added in all tubes , as well as one drop of reagent **2** (Urease) . Add 10 µl of reagent **(1)** in standard tube, and 10 µl of serum to sample tube. Mix and incubate at least 3 minutes at 37 °C or 5 minutes at 20-25 °C ,then

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200 µl of reagent(**4**) were added to all tubes . Mix and incubate for 5 minutes at 37 °C.Measure absorbanc at 580 nm of sample (A sample) and standard (A standard) against reagent blank.

Calculation:

$$\text{Urea concentrat ion} = \frac{\text{A sample}}{\text{A standard}} \times n$$

Where n = 8.33 m mol/L
n = 50.0mg/dL

6- *Creatinine:*

As reported by **Henry (1974)**, creatinine in alkaline solution reacts with picrate to form a colored complex. The rate of formation of the complex is measured photometrically at 492 nm.

Reagents:

Reagent(1): Creatinine standard (2.0mg/dL).
Reagent(2): Color reagent (Picri acid: 38 mmol/L).
Reagent(3): Alkaline reagent (sodium hydroxide 0.4mmol/L).

Procedure:

According to requirements, working solution is prepared by mixing reagent (2) and reagent (3) in the ratio 1:1. This solution stable for 6 hours at 20-25 °C, when stored in a dark bottle. Pipette into test tube or cuvette 1.0 ml of working solution and 100 µl of standard (reagent 1) or sample (serum). Mix. after 30 seconds read initial absorbance. (A**1**) of the standard or sample. Exactly 2 minutes later, read absorbance (A**2**) of the standard or sample.

Calculation

$$A_2 - A_1 = A_{\text{standard}}$$

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Concentration of creatinine in serum

$$\text{creatinine (mg/dL)} = \frac{\text{A sample}}{\text{A standard}} \times 2$$

7-Bilirubin

Bilirubin is coupled with diazotized sulfanilic acid in the presence of caffeine to give an azo dye. Normal saline is used instead of caffeine when direct Bilirubin is determined (**Jendrassik** ***et.al.,*** **1938**).

Reagents:

Reagent(1): Sulfanilic acid (31.0 mmol/L), HCl (0.2N).
Reagent(2): Sodium nitrite (28.0 mmol/ L).
Reagent(3): Caffeine (28.0 mol/L), sodium benzoate (0.55 mol/L).
Reagent(4): Tartrate (0.99 mol/L.), NaOH (2.0N).

Procedure:

A- *Total Bilirubin:*

For two tubes labeled as sample blank and sample, 200µl of reagent (1) were added for both. One drop of reagent (2) was added to sample tube 1.0 ml of reagent (3) was added for both tubes + 200µl of sample (serum should be separated immediately). Mix and incubate for 5 minutes at 20-25 °C. Measure the absorbance of sample (A_{sample}) at 578 nm against sample blank.

B- *Direct Bilirubin*:

For two tubes labeled as sample blank and sample add 200 µl of reagent (1). One drop of reagent 2 was added to sample tube. 2.0 ml of saline (0.9% NaCl) + 200 µl of sample were added for both tubes. Mix and incubate for exactly 5 minutes at 20-25 °C. Measure absorbance of sample (A sample) at 546 nm against sample blank.

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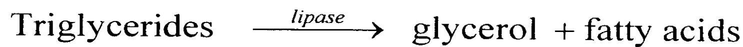
Calculation:

Total Bilirubin $= A_{sample} \times 10.8$

Direct Bilirubin $= A_{sample} \times 14.4$

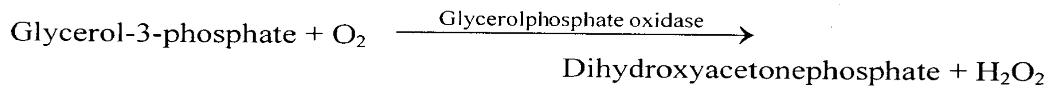
8-*Triglycerides*:

Triglycerides were determined by the enzymatic colorimetric method according to **Trinder(1969)** . The principles of the measurements of triglycerides are based on the following equations.

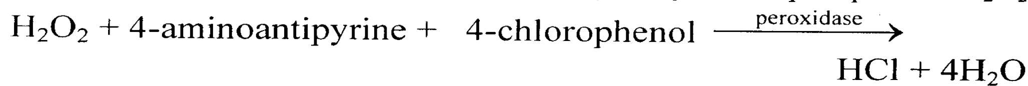


$$\text{Triglycerides} \xrightarrow{lipase} \text{glycerol} + \text{fatty acids}$$

$$\text{Glycerol-3-phosphate} + \text{ADP} \xrightarrow{\text{Glycerokinase}} \text{Glycerol} + \text{ATP}$$



$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{Glycerolphosphate oxidase}} \text{Dihydroxyacetonephosphate} + H_2O_2$$



$$H_2O_2 + \text{4-aminoantipyrine} + \text{4-chlorophenol} \xrightarrow{\text{peroxidase}} HCl + 4H_2O$$

After enzymatic hydrolysis of triglycerides, the reaction product, hydrogen peroxide, forms with 4- aminoantipyrine and 4- chlorophenol under the catalytic influence of peroxidase a red violet indicator (quinoneimine).

The increase of O.D. of this indicator measured at 546 nm is proportional to the triglycerides concentration in the sample.

Reagents:

Reagent(1): Standard (200mg/dl)

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Reagent(2): *Color reagent*: 4-chlorophenol (6 mmol/L); Mg – acetate (5 m mol/L), tris buffer (50 mmol/L), 4-aminatipyrine (1.0 mmol/L), ATP (1.0 mmol/L), Lipases (≥100 U/ml), Glycerolkinase (≥120 U/ml), Glycerol-3-phosphate oxidase((≥2.5 U/ml) and peroxidase (≥4.0 U/ml).

Procedure:

For tubes labeled as blank , standard and sample , 10 µl standard (R1) is added to standard tube ; 10 µl of sample is added to sample tube , then 1 ml of color reagent (R2) were added for all tubes , Mix and incubate for 10 minutes at 20-25 °C . Measure absorbance of sample (A_{sample}) at 500 nm and standard within 60 minutes.

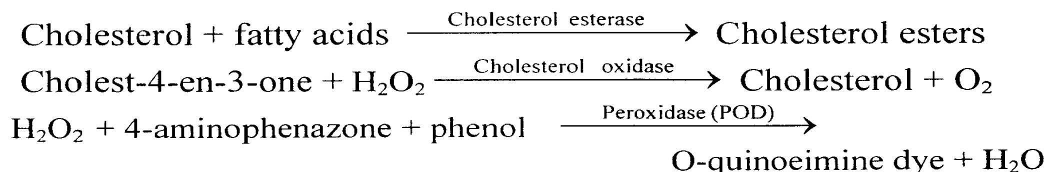
Calculation:

$$\text{Triglycerides conc. (mg/dl)} = \frac{\text{A sample}}{\text{A standard}} \times 200$$

9- *Cholesterol*

Cholesterol was determined by enzymatic colorimetric method according to **Richmond (1973), Roeschlau *et al.* (1974) and Trinder (1969).**

The principle of enzymatic colorimetric determination of Cholesterol based on the following equations:



$$\text{Cholesterol + fatty acids} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol esters}$$

$$\text{Cholest-4-en-3-one} + H_2O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholesterol} + O_2$$

$$H_2O_2 + \text{4-aminophenazone + phenol} \xrightarrow{\text{Peroxidase (POD)}} \text{O-quinoeimine dye} + H_2O$$

After enzymatic hydrolysis and oxidation of cholesterol, the reaction product, hydrogen peroxide forms with 4-aminophenazone and phenol in the

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presence of peroxidase a red violet indicator (quinoneimine). The increase of O.D of this indicator measured at 564 nm is proportional to the cholesterol concentration in the sample.

Reagents:

Reagent(1): Standard (200mg/dl)
Reagent(2): *Color reagent: phenol* (4mmol/L); tris buffer (50mmol/L); detergent (0.2%).
Cholesterol esterase (≥160U/L); cholesterol oxidase (≥120 U/L); peroxidase (≥2000U/L); and 4-aminoantipyrine (0.45m mol/L).

Procedure:

For tubes labeled as blank , standard and sample , 10 µl of standard (R1) is added to standard tube ; 10 µl of sample is added to sample tube , then 1 ml of color reagent (R2) was added for all tubes , Mix and incubate for 10 minutes at 20-25C . Measure absorbance of sample (A_{sample}) at 500 nm and standard ($A_{standard}$) within 30 minutes.

Calculation:

$$\text{Cholesterol conc. (mg/dL)} = \frac{\text{A sample}}{\text{A standard}} \times 200$$

10- *HDL – Cholestero:l*

Low density lipoproteins (LDL and very LDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphotungestic acid in the presence of magnesium ions. After centrifugation, the cholesterol concentration in the HDL (high density lipoproteins) fractions, which remains in the supernatant, is determined (**Trinder, 1969; Richmond, 1973 and Assmann, 1979**).

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Reagents (precipitant):

Phosphotungestic acid 13.9 mol/L.
Magnesium chloride 570 mmol/L.

Procedure:

Precipitation:

In test tube add 500 µl of sample + 50 µl of precipitat. Mix and allow standing 10 minutes at room temperature centrifuge for 10 minutes at 4000 rpm. Separate off the clear supernatant within two hours and determine the cholesterol content by the method previously described using Reagent 2 (color reagent).

For test tube labeled as blank and sample, add 1000 µl of reagent (2) for both tubes. Then add 50 µl of sample to sample tube and 50 µl of distilled water to blank tube. Mix and incubate for 10 minutes at 20-25 °C Measure absorbance of sample (A_{sample}) at 520 nm with in 30 minutes.

Calculation:

1- HDL –cholesterol

Concentration of HDL –cholesterol in supernatant:

$$\text{In mg/dL} = A_{sample} \times 180$$

2- LDL –cholesterol

$$\text{In mg/dL} = \text{total cholesterol} - \frac{triglycerides}{5} - \text{(HDL cholesterol)}$$

Statistical Analysis:

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Data obtained were subjected to statistical analysis as shown by **Snedecor and Cocharn (1981).** Mean values of treatment were compared using LSD at 5% level.

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RESULTS AND DISCUSSIONS

The current study was designed to investigate and to assess the protective effects of natural antioxidants on some heavy metal toxicity. Thus cadmium and lead have been used to induce toxicity in rats and to study the therapeutic efficacy of some natural antioxidants i.e. DL –methionine and ascorbic acid administrations aftermath of such heavy metal exposure. A dose of 300ppm of lead as lead acetate and 40 mg cadmium as cadmium chloride through drinking water for five weeks has been used to study weekly body weight of female rats and its organs as well as some biochemical parameters in blood serum. It is worth to note that the levels of Cd or Pb treatments used in this study correspond to human occupational exposure to both heavy metals, or environmental exposure in heavily contaminated areas **(WHO, 1992).**

I .1. Body weight:

Body weight and relative body weight gain (%) of seven groups are given in table (1) and illustrated in figures (1, 2) through five weeks. Results showed clearly that four weeks of cadmium or lead exposure at such levels reduced the mean weekly body weight, but the animals did not show overt clinical signs of toxicity. It might be due to the lower dose or less duration of exposure to induce overt clinical sign of toxicity. In general, the relative body gain percentage of control rats (group I) was 9.02%, while it were 6.28 and 6.45% for lead (group II) and cadmium (group V) respectively. However, results also showed that there was a steady increase in body weight of rats of all the groups and the growth was comparatively poor in heavy metals exposed rats. The beneficial role of antioxidant i.e. methionine or ascorbic

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Table (1): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on weekly body weight during the entire period of the experiment (5 weeks) of female rats.

Treatments	Weeks / body weight in gram.					
	1 (Initial)	2	3	4	5 (Final)	Relative body gain %
Group I Control	134.0* ±3.1	136.1 ±4.0	139.8 ±2.9	141.1± 3.6	146.1 ±2.7	9.02
Group II Pb	135.2 ±2.9	137.4 ±4.2	139.1 ±3.0	141.1± 3.0	143.7 ±3.5	6.28
Group III Pb+ Met	137.5 ±3.0	139.5 ±3.9	141.1 ±3.5	144.5± 4.0	147.9 ±3.1	7.56
Group IV Pb+ Vit.C	139.5 ±2.1	144.2 ±3.1	146.0 ±2.8	148.0± 3.2	150.1 ±3.5	7.59
Group V Cd	133.2 ±3.1	134.5 ±3.7	136.8 ±3.2	138.5± 2.9	141.8 ±4.0	6.45
Group VI Cd+ Met	137.8 ±3.5	139.5 ±3.0	141.7 ±4.0	143.7± 3.5	147.9 ±4.2	7.32
Group VII Cd+Vit.C	134.8 ±2.7	136.4 ±4.5	139.1 ±3.2	141.2 ±3.2	144.5 ±3.5	7.19

* All values are means ±SD of 7 animals

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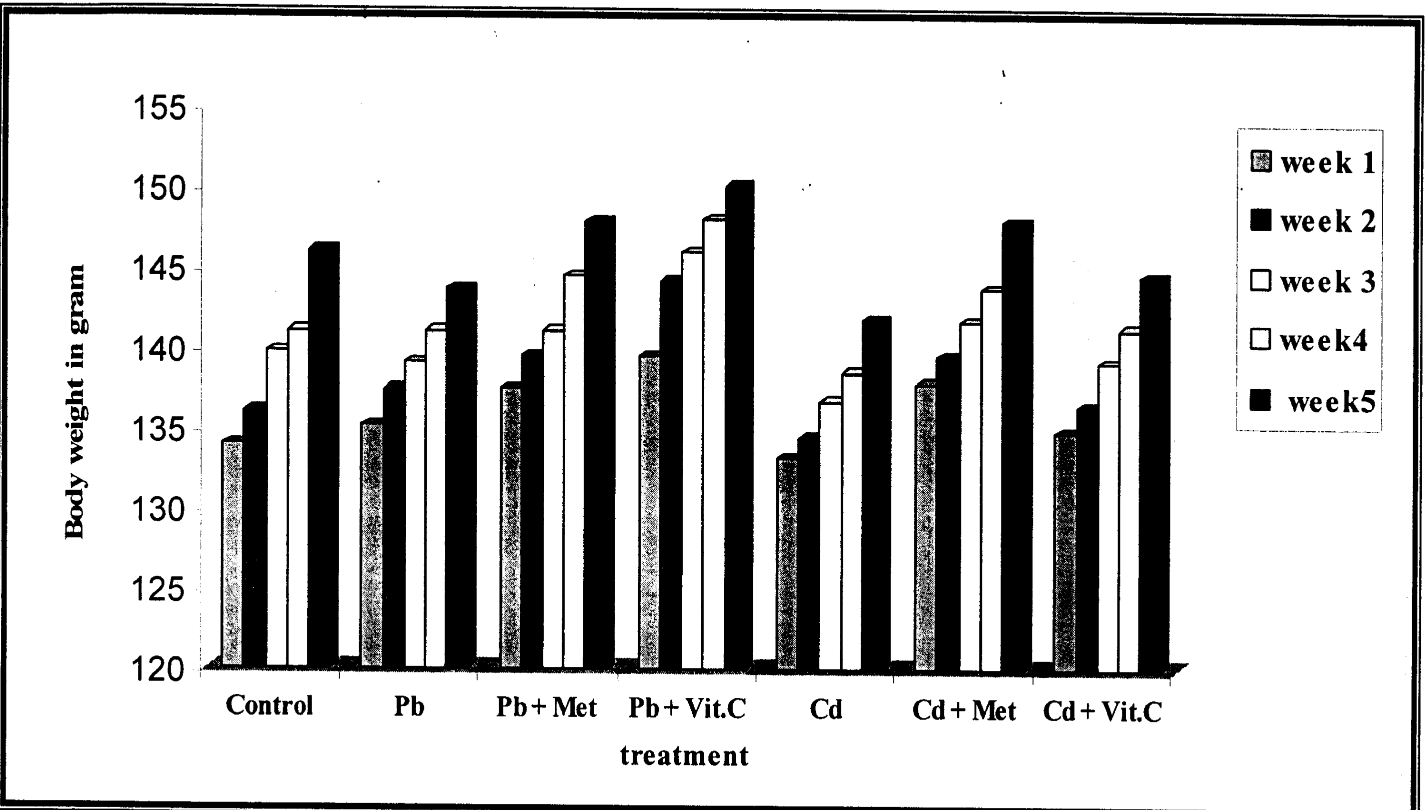

Figure (1): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on weekly body weight during the entire period of the experiment (5weeks) of female rats.

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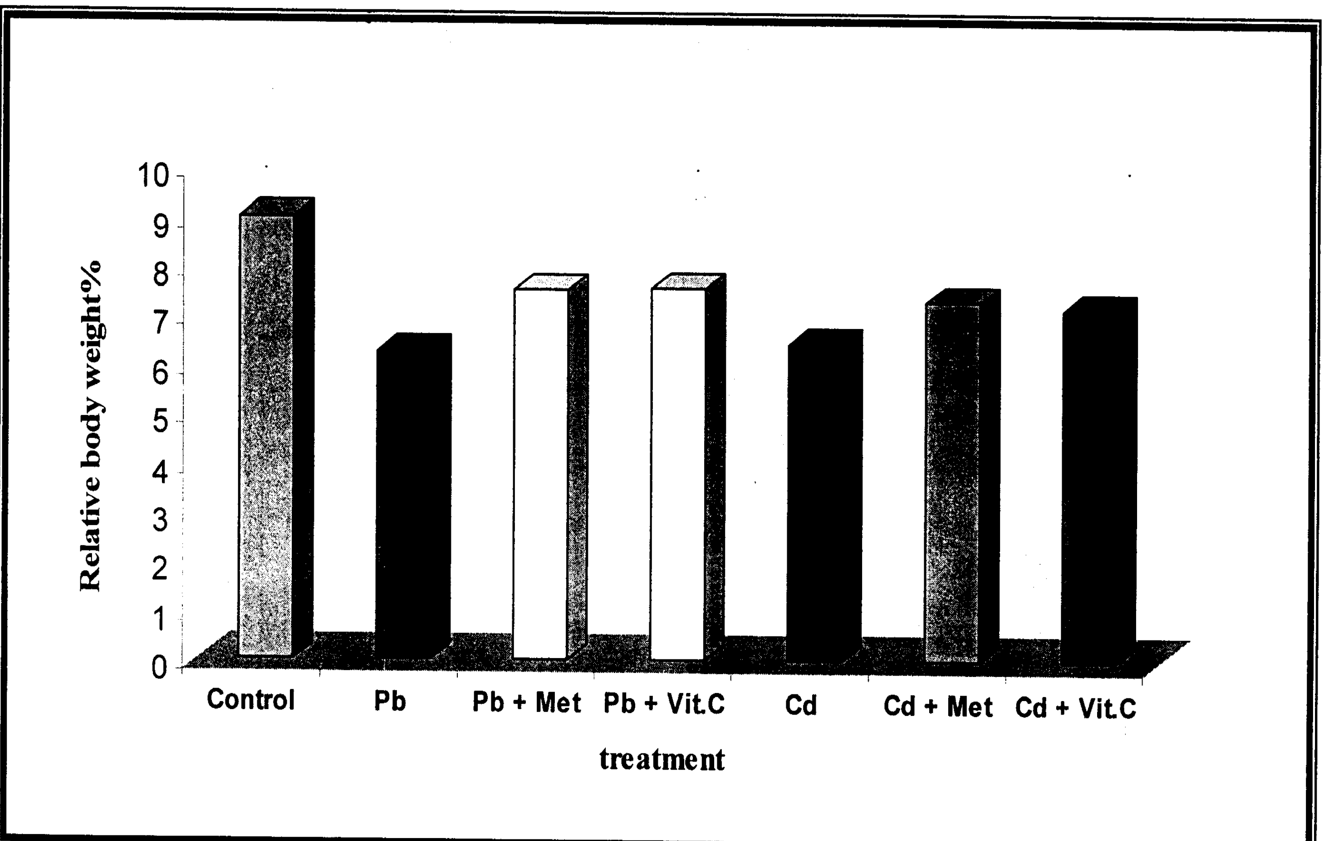

Figure (2): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on Relative body weight gain % during the entire period of the experiment (5 weeks) of female rats.

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acid with heavy metal treatments was observed , where the relative body gain percentage of groups III Pb + Met (7.56) , IV Pb + Vit.C (7.59),VI Cd + Met (7.32), VII Cd + Vit.C(7.19) were almost high as compared to the values of lead- (6.28) or cadmium – (6.45) exposed rats (groups II and V) . The present data can be considered in accordance with others, where in this concern, **Grant *et al.*, (1980)** found a reduction in body weight with Pb dose of 250mg /L, where as **Hayashi (1983)** found that a Pb of 500 mg / L did not alter body weight of rats. **Cropas *et al.*, (2002)** reported that Pb intoxication (Pb: 300mg/L) had no effect on weight gain or on water consumption in rat mothers during 3 weeks of gestation.

The beneficial role of antioxidants against heavy metals have been reported by **Fariss (1991)** who proved that antioxidant are useful in protecting against Cd toxicity. **Petra *et al.*, (2001); Patra and Swarup (2004) and Nandi *et al.*, (2005)** reported the beneficial role of ascorbic acid **and** methionine against Pb toxicity using rats as experimental animals. However, **Flora and Tandon (1986)** reported the significant ameliorative efficacy of ascorbic acid and methionine in experimental lead toxicity in rats when given simultaneously as compared to post exposure treatments.

I .2. Rat organs weight:

Data presented in table (2) and illustrated in figures (3-7) show the effect of heavy metals i.e. lead and cadmium as well as natural antioxidants i.e. methionine and ascorbic acid on rat organs weight: liver (fig.3), kidney (fig.4), lung (fig.5), spleen (fig.6), and heart (fig.7). Results showed that all rat organs weight was significantly affected by heavy metal intoxification as compared to control group.

However, the higher weight of both liver (8.14g: group II and 7.94g: group V) or kidney (1.32g: group II and 1.33g: group V) of exposed animals could be attributed to the fact that both organs accumulate Pb and Cd

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Table (2): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on rat organs weight.

Treatments	Organs (gram).						
	liver	R.L.W	kidney	R.K.W	lung	spleen	heart
Group I Control	6.85 *±1.14	4.68	1.24 ±0.21	0.848	1.34 ±0.32	0.94 ±0.30	0.66 ±0.23
Group II Pb	8.14 ±1.11	5.66	1.32 ±0.07	0.918	1.49 ±0.25	0.79 ±0.19	0.60 ±0.11
Group III Pb+ Met	7.15 ±0.97	4.83	1.29 ±0.12	0.872	1.44 ±0.21	0.79 ±0.15	0.65 ±0.08
Group IV Pb+ Vit.C	7.60 ±0.81	5.06	1.30 ±0.13	0.886	1.25 ±0.26	1.16 ±0.17	0.62 ±0.17
Group V Cd	7.94 ±0.99	5.59	1.33 ±0.11	0.937	1.49 ±0.24	0.86 ±0.10	0.66 ±0.17
Group VI Cd+ Met	7.25 ±0.83	4.90	1.28 ±0.20	0.865	1.32 ±0.14	1.01 ±0.14	0.60 ±0.04
Group VII Cd+Vit.C	7.34 ±0.43	5.07	1.29 ±0.13	0.892	1.41 ±0.18	1.16 ±0.21	0.68 ±0.04
Mean	7.424	-	1.293	-	1.339	0.958	0.645
L.S.D 0.05	0.921	-	0.165	-	0.274	0.207	0.154

* All values are means ±SD of 7 animals.

R.L.W: Relative liver weight = % of body weight
R.K.W: Relative kidney weight = % of body weight

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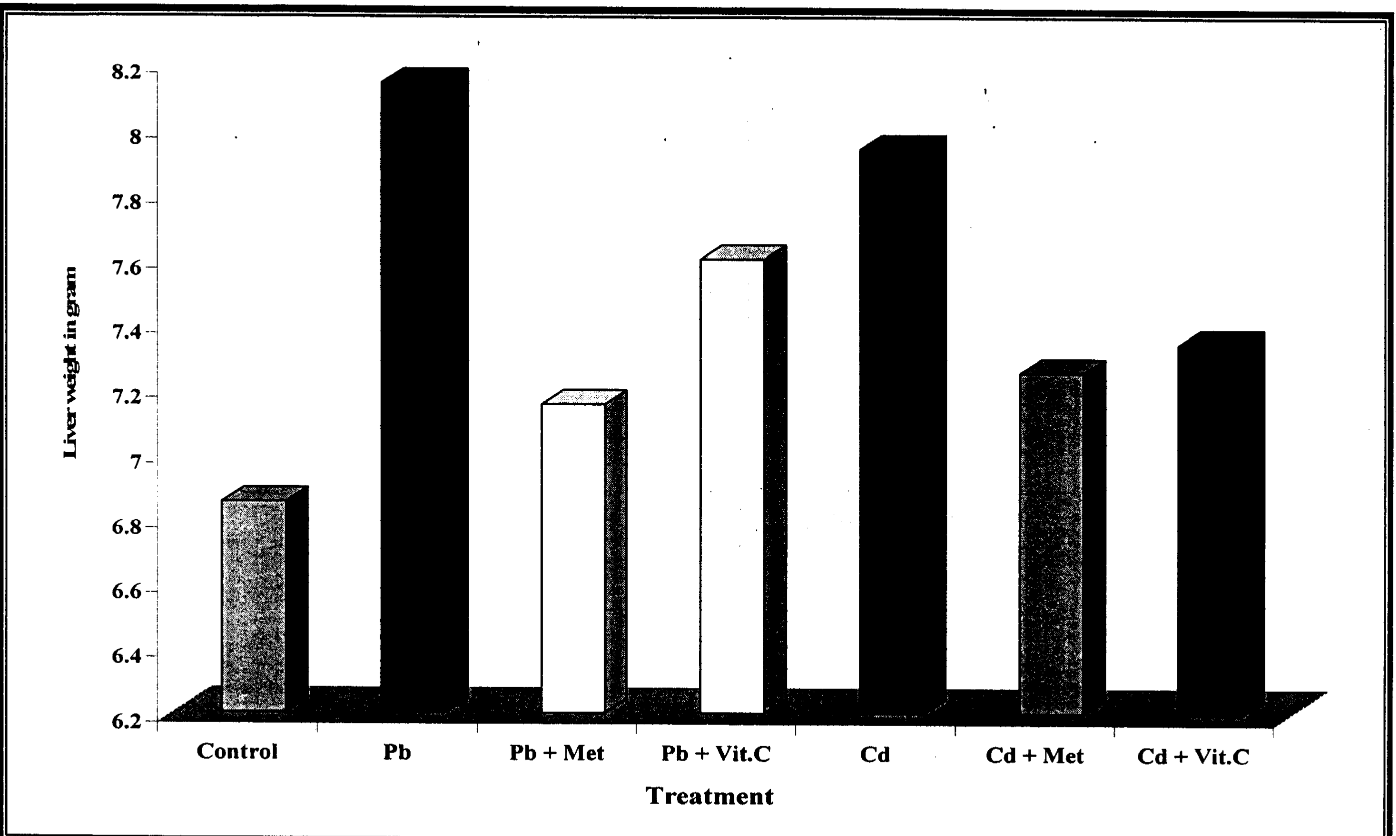

Figure(3): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on rat liver weight (in gram).

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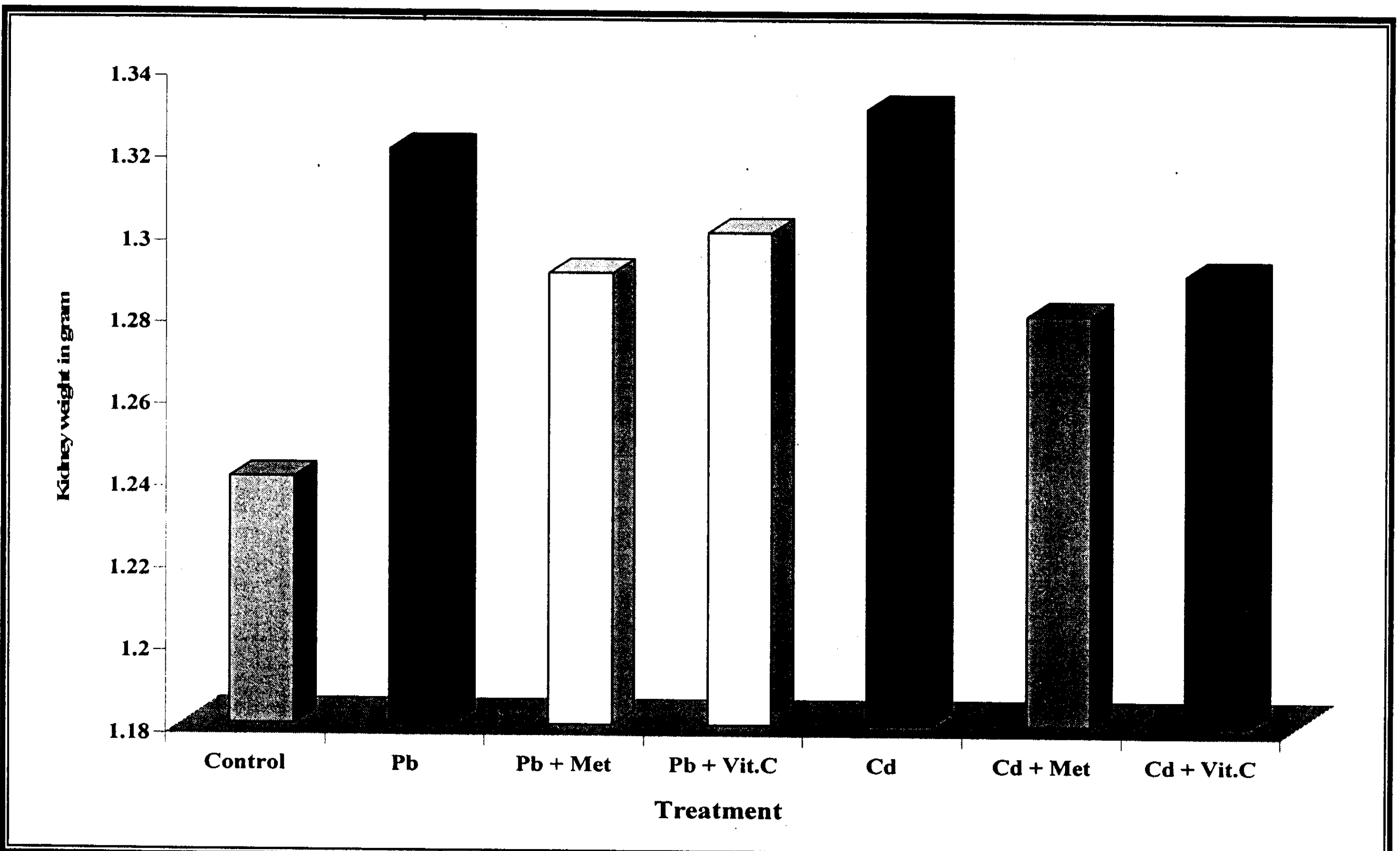

Figure(4): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on rat kidneys weight (in gram).

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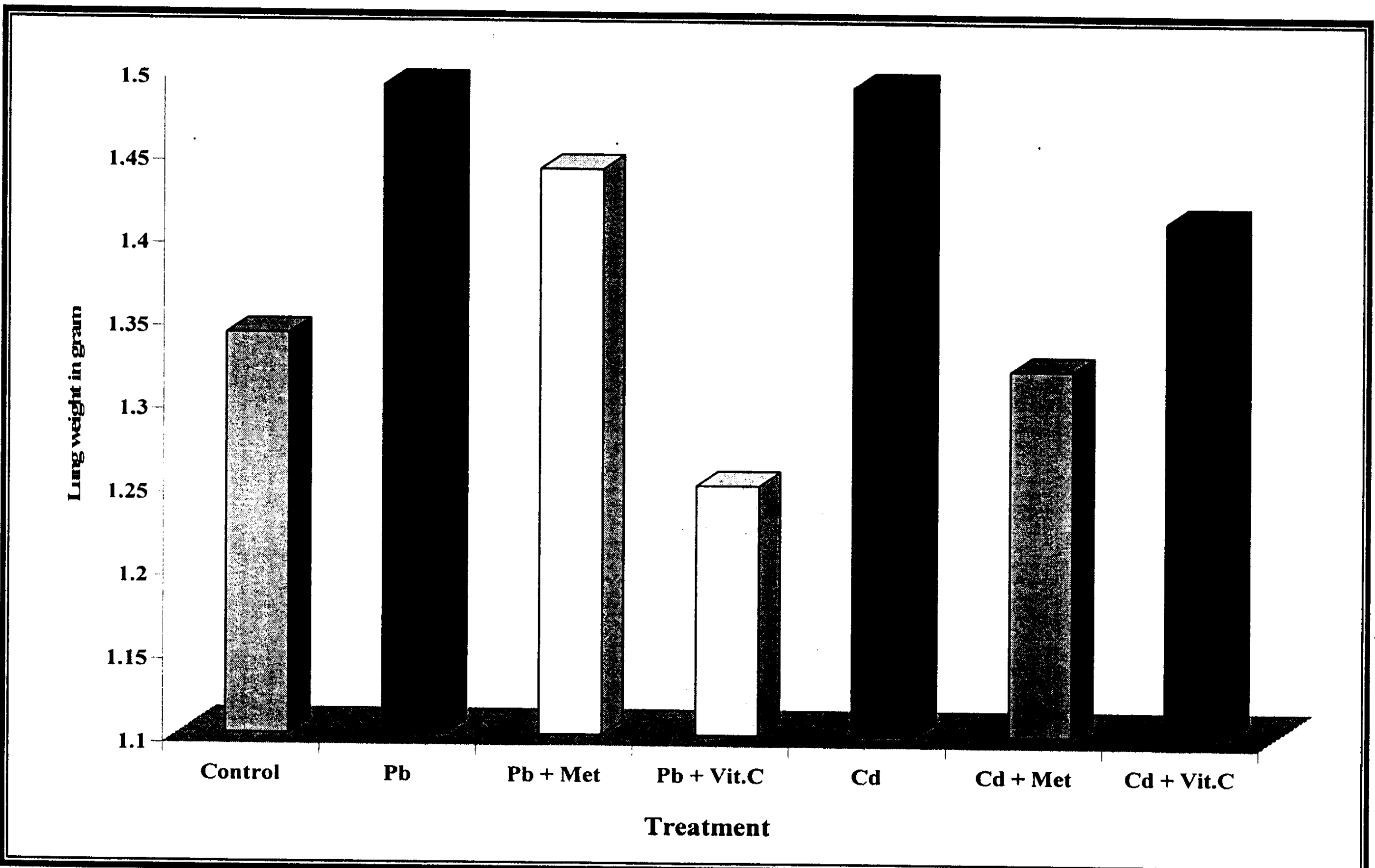

Figure(5): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on rat Lungs weight (in gram).

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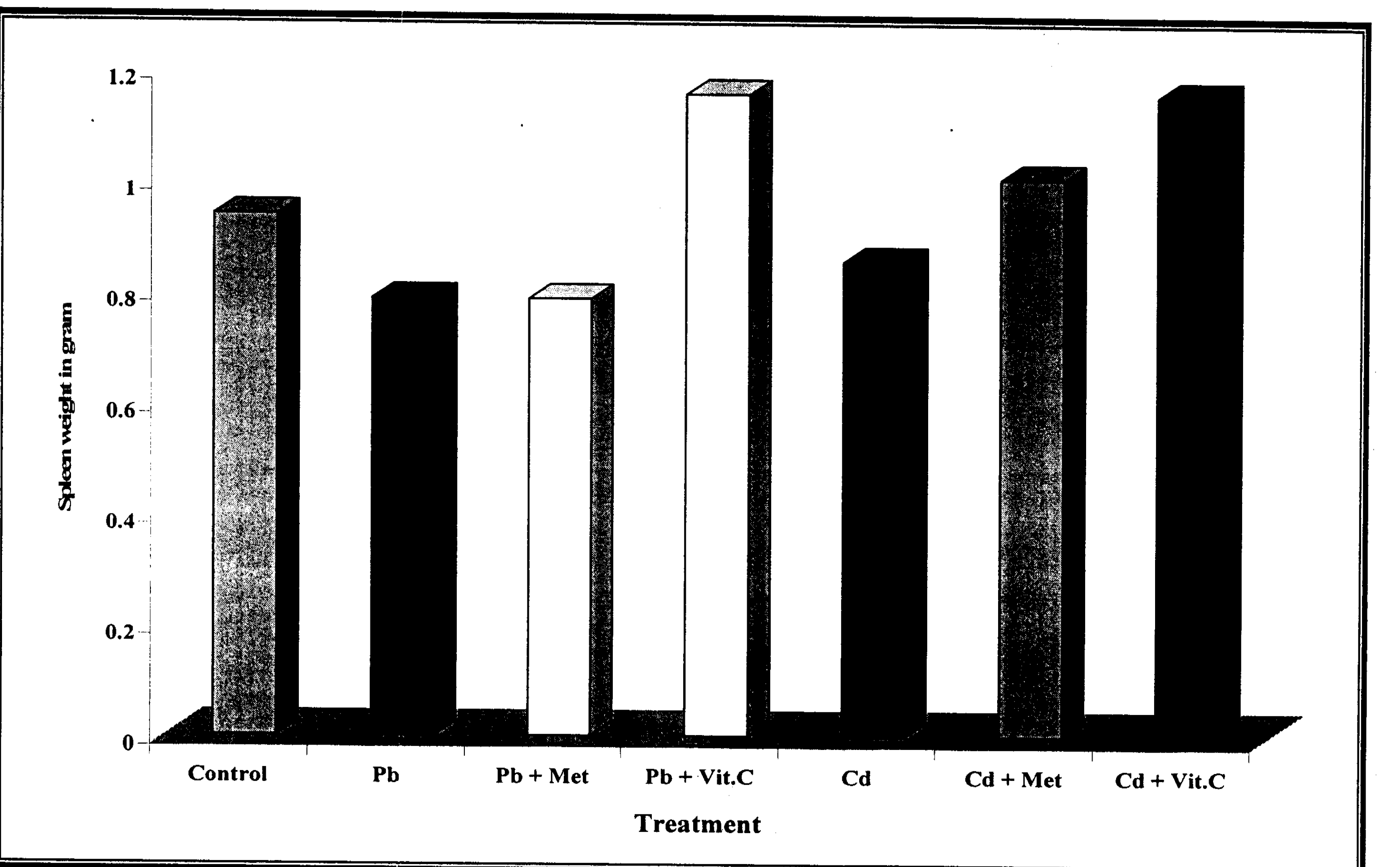

Figure (6): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on rat spleen weight (in gram).

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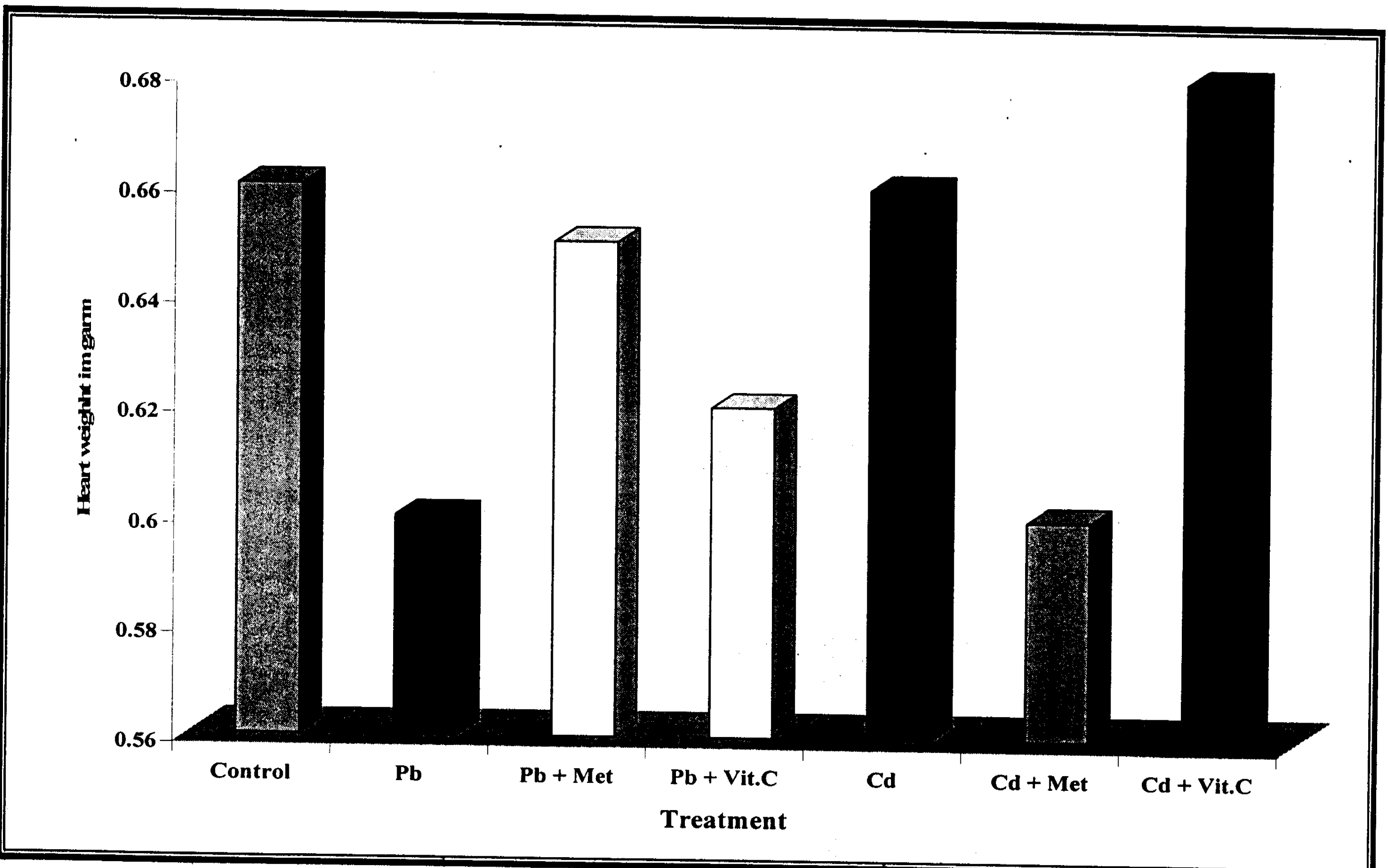

Figure(7): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on rat heart weight (in gram).

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(respectively). Both organs are target for heavy metals accumulation. Such results are confirmed especially when compared with control group (6.85g and 1.24 g) for liver and kidneys respectively. Similar results were observed for the relative liver weight and relative kidneys weight.

On the other hand, results also showed that supplementation of antioxidants i.e. methionine and ascorbic acid in diets of exposed animals seemed to reduce liver and kidneys weight as compared to exposed animals without antioxidant supplementation.

In this concern, **Friberg *et.al.* (1979)** reported that 90% of the total body lead is contained within bones. Blood account for 4% and the remaining lead resides mainly in the liver and kidneys (**Boeckx, 1986**). The liver and kidneys are also known to play a major role in the elimination of lead (**Goyer and Cherian, 1979**) and hence account for the toxic actions (**Lockitch , 1993**).

Results also showed that lung, spleen and heart weight were affected significantly following Pb or Cd administration with no definite trend.

II. Biochemical parameters.

II.1. Indicators of renal function

II.1.1. Serum total protein:

Liver and kidneys are important organs of metabolism, detoxification storage and excretion of toxic substances and their metabolites are specially vulnerable to damage.

Serum total protein is one of renal function indicators. However, data presented in table (3) and illustrated in figure (8) showed that a dose of 300 mg of lead as acetate, or 40 mg of cadmium as $CdCl_2$ through drinking water

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Table (3): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb andCd) on biochemical indicators of renal function of female rat serum.

Treatments	Total protein g /dL	Urea mg/dL	Creatinine mg/dL
Group I Control	5.86* ±0.42	35.17 ±0.82	0.53 ±0.05
Group II Pb	5.10 ±0.27	46.91 ±0.73	0.63 ±0.04
Group III Pb+ Met	5.70 ±0.45	41.60 ±0.96	0.59 ±0.03
Group IV Pb+ Vit.C	6.15 ±0.35	42.50 ±0.79	0.60 ±0.02
Group V Cd	5.21 ±0.32	49.70 ±1.24	0.79 ±0.05
Group VI Cd+ Met	6.63 ±0.41	39.32 ±0.78	0.72 ±0.03
Group VII Cd+Vit.C	6.70 ±0.53	40.56 ±1.16	0.70 ±0.03
Mean	5.887	42.250	0.651
L.S.D 0.05	0.462	1.044	0.040

* All values are means ±SD of 7 animals

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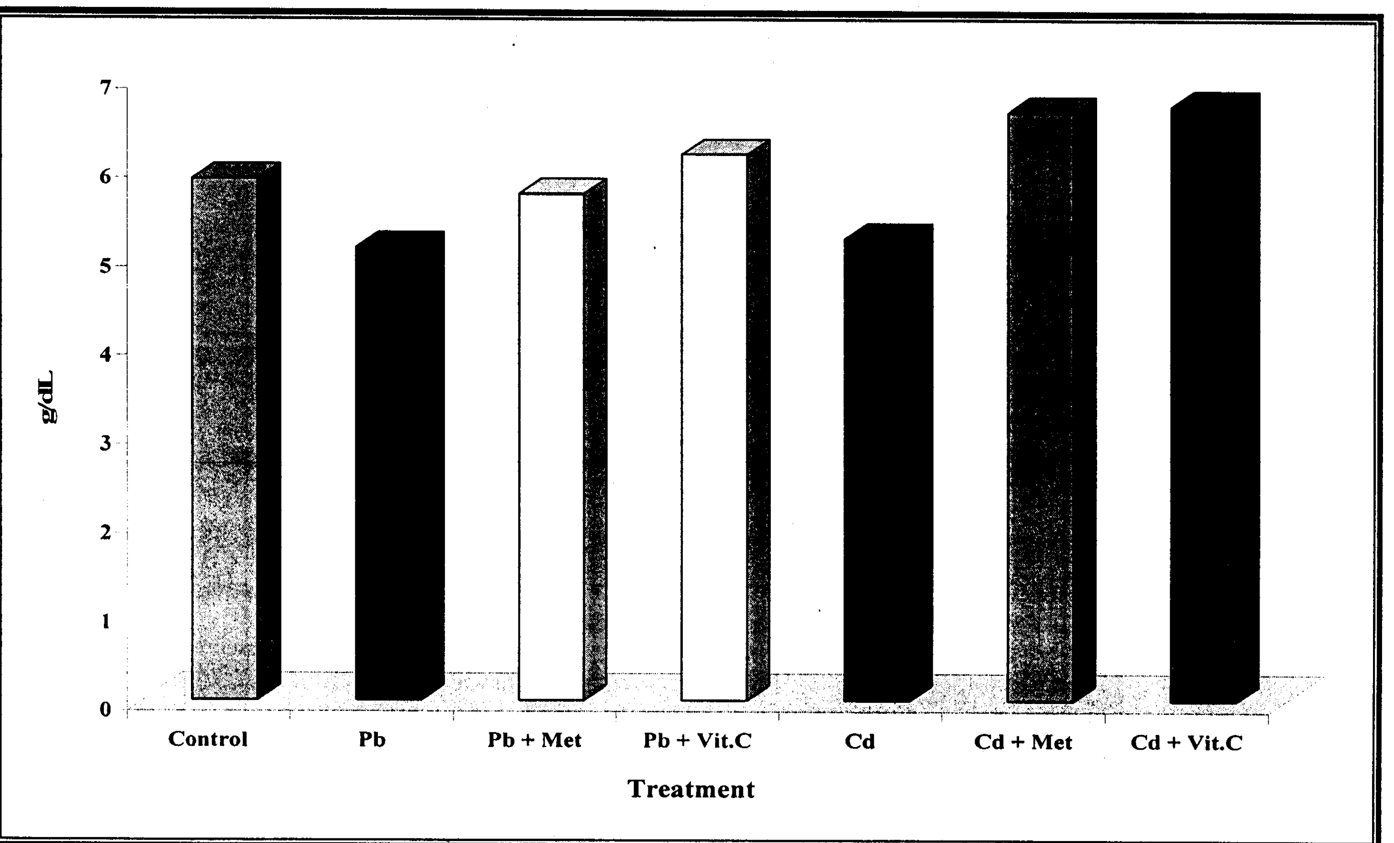

Figure(8) : Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on Total protein in serum (g /dL) of female rats.

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for 5 weeks had a significantly lower serum total protein of exposed rats (5.10and 5.21g/dL respectively) than rats drank distilled water (5.86 g/dL).

Concering the effects of antioxidant administration in experimental lead or cadmium toxicity in rats, data showed that methionine treatment with lead toxicity could not increase serum total protein (5.70 g/dL), but it increased such parameter (6.63 g/dL) when it administered with cadmium toxicity. On the other hand, ascorbic acid administration showed more efficient, where it increased serum total protein in both exposed -Pb or -Cd rats (6.15and 6.70 g/dL respectively).

Many studies have explored the mechanisms and symptoms of lead toxicity, recent studies reported Pb as a potential agent for inducing oxidative stress by the reactive of oxygen species (ROS) , **Gurer and Ercal (2000)**. Kidneys are known to play a major role in the elimination for the toxic actions (**Lockitch, 1993**). However, **Ding *et al.*, (2000)** reported that meso 2,3-dimercaptosuccinic acid like methionine (sulphur-containing amino acid) has a high therapeutic index , which scavenges ROS , and has been identified as potentially useful drug for the treatment of lead poisoning (**Graziano *et.al.* , 1983).** Also, **Lewis and Wills (1962)** suggested that peroxide formation may lead to oxidative destruction of thiol groups of amino acid and proteins. Moreover, **Sivaprasad *et al.*, (2004)** reported that ROS may modify proteins, lipids and DNA. As reported by **Goering (1993) and Radwanska-Konarzewska *et al.*, (1993),** lead can perturp protein biosynthesis in hepatocytes, including the structural protein. Moreover, **Needleman and Bellinger (1991)** showed that Pb can interact with some amino acids (Cys, Asp, Glu, Tyr) of enzymes and it might alter protein synthesis and produce a decrease in total protein amount.

On the other hand, **Brzoska *et al.*, (2003)** reported that Cd exposure affected some biochemical markers of kidney function, but it had no effect on the total protein concentration in serum. Meanwhile, **Ziegler (1985)** suggested

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that high binding affinity of Cd for protein and sulfhudryl groups is considered to account for most of its numerous toxic effects.

Brzoska *et al.*, (2003) reported that exposure to Cd alone had no effect on the total protein concentration in serum .**Aughey *et al.*, (1984)** noted early pathological changes in rat kidney already after 6 weeks of administration of 50mg Cd/L in drinking water.

It is worth to note that albumin constitutes about 60% of total serum protein, which has important binding and transport function. It binds and inactivates substances including calcium, bilirubin, fatty acids, urate and hormones. When albumin levels are reduced, toxic effects can develop from an increase in unbound substances.

II.1.2. Urea in serum:

Urea is the main waste product of protein breakdown. It is formed in the liver and excreted by the kidneys. So, urea test is a test of renal function.

However, data presented in table (3) and illustrated in figure (9) showed that Pb or Cd exposure increased significantly the concentration of urea in serum (46.91 and 49.70mg/dL respectively) by 33.3 % and 41.31% respectively as compared with control group (35.17 mg/dL). Similar results were reported by **Brzoska *et al.*, (2003)** for Cd. On the other hand, **Shibutani *et al.*, (2001)** reported that Cd activity causes renal tubule damage and then glomerular filtration impairment, and this may account for the increase of urea concentrations of animals receiving $CdCl_2$.

Data also showed that Pb toxicity led to an increase of urea concentration in the blood, suggesting the inability of the kidney to excrete it.

As regarded with the effect of antioxidants to eliminate heavy metals, the obtained results showed that methionine reduced the concentrations of urea in serum of Pb- or Cd – exposed rats (41.60 and 36.32 mg /dL respectively). The beneficial role of ascorbic acid was also observed in the

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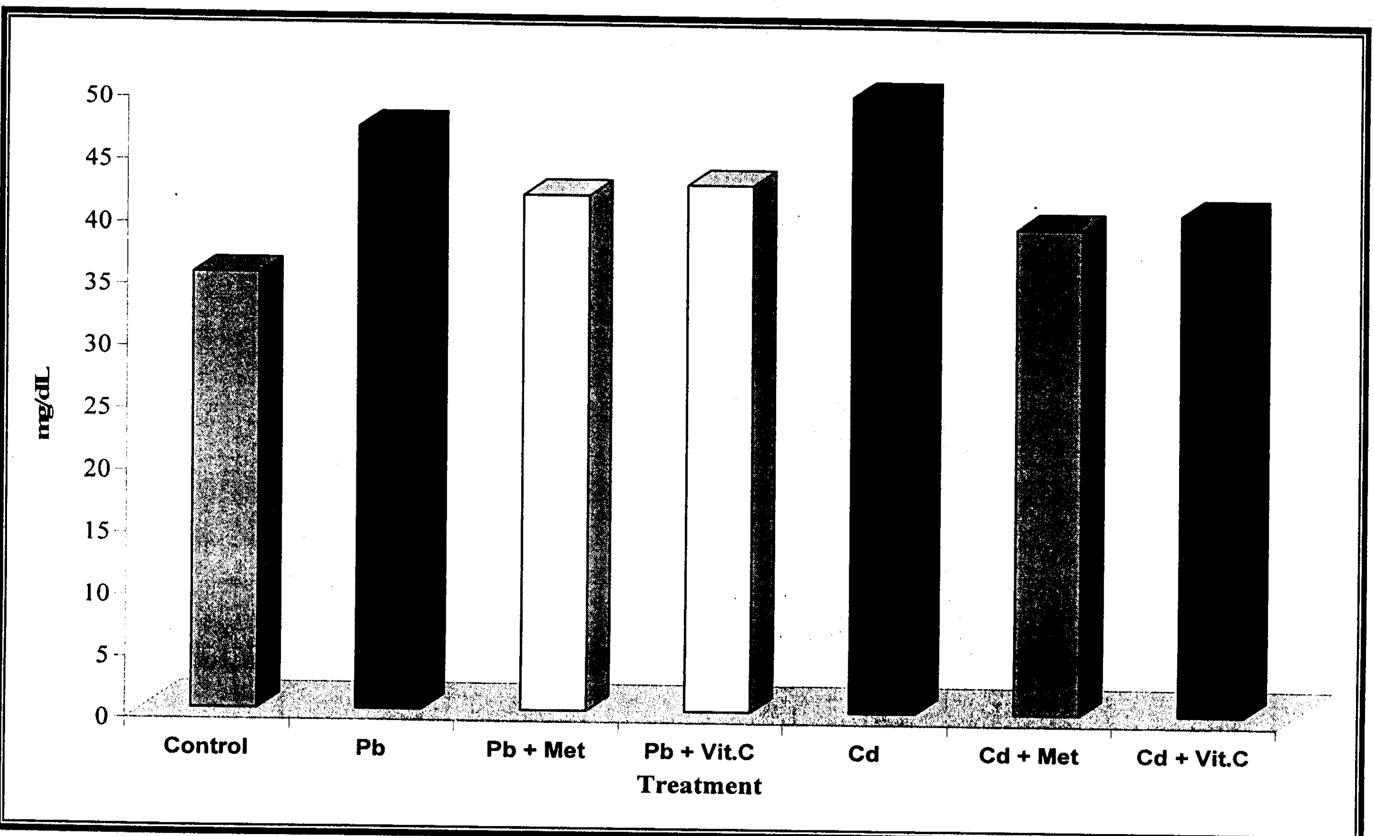

Figure (9): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on Urea in serum (mg/dL) of female rat

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present study where it reduced the levels of urea in serum (42.50and 40.56 mg/dL). Similar results were obtained by others, **Reed and Orrenius (1997)** reported that methionine a precursor of amino acid glutation synthesis protects cells from oxidative damage and plays vital role in detoxification of xenobiotics. **Patra *et al.*, (2001) and patra and Swarup (2004)** reported the beneficial role of ascorbic acid and methionine alone or with chelator against Pb toxicity. **Kowalczyk *et al.*, (2003)** reported that concentration of urea was increased in blood serum as a result of Cd administration. **Brzoska *et al.*, (2003)** reported that exposure to Cd alone increased the serum urea concentration by 16%.

II.1.3. Creatinine in serum:

Creatinine is a nitrogenous waste product formed from the metabolism of creatine in skeletal muscle. Creatinine difuses freely through the body. It is filtered from the extracellular fluid by the kidney and excreted in the urine. The excretion of creatinine is mainly renal, and in the absence of disease or toxicity, relatively constant. Measurement of serum creatinine is an important test of kidney function. However, the obtained results presented in table (3) and illustrated in figure (10) showed clearly that the levels of creatinine in serum produced druing the intoxication of Pb or Cd in rats exhibited higher and significant increase in blood serum of rats fed with Pb or Cd in drinking water (0.63: group II and 0.79: group V mg/dL respectively) as compared to control group: I fed with drinking water (0.53 mg/dL). It is worth to note that the presence of increasing creatinine concentration in the blood suggests the inability of kidney to excrete this product, which further suggests a decrease (fall) in glomerular filtration rate as a result of heavy metals administration **Davis and Berndt, (1994) ; Shinutani *et al.*, (2001); Kowalxzyk *et al.*, (2003).**

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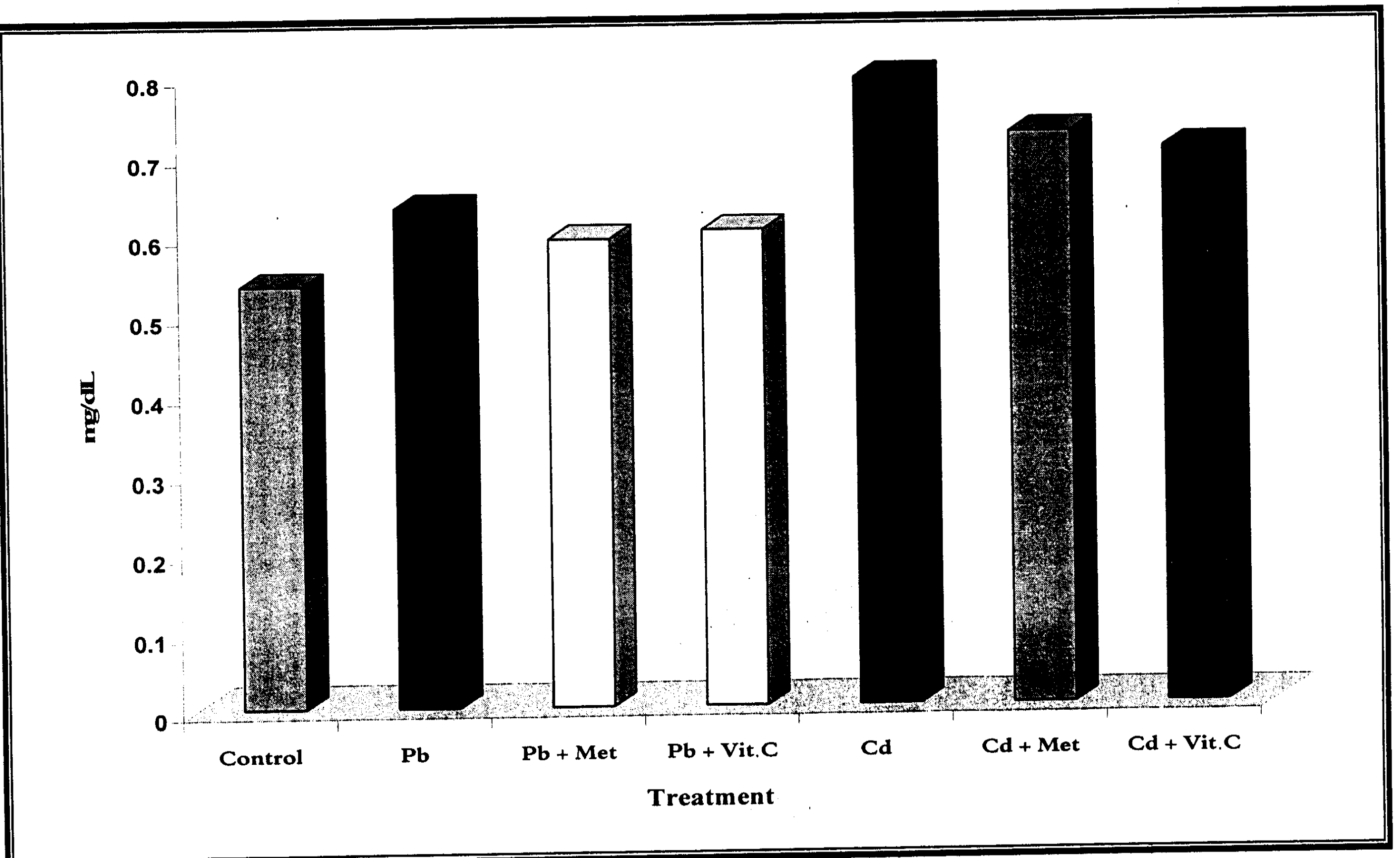

Figure (10): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on Creatinine in serum (mg/dL) of female rats

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On the other hand, results also showed that the antioxidants i.e. methionine and ascorbic acid eliminated the effects of Pb toxification, where such antioxidants protected cells from oxidative damage and played good role in detoxification of such heavy metal (serum creatinine concentration was 0.59: group III and 0.60:group IV mg/dL respectively). The oxidative damage of Cd seemed higher than those of Pb, where serum creatinine concentration was 0.72: group VI and 0.70 group VII mg-dL in rats administered Cd in drinking water and received methionine or ascorbic acid in diets. Such results are in good accordance with the finding of **Patra *et al.*, (2001) and Ramanthan *et al.*, (2002)** who reported that antioxidants have been beneficial to mitigate chemical induced oxidative damage.

II.2.Indicators of liver function:

II.2.1. Glucose in serum:

Glucose provides the energy of life process .It is the main end product of carbohydrate digestion.

Data presented in table (4) and illustrated in figure (11) showed clearly that Pb or Cd administrations altered glucose metabolism where blood serum glucose levels of both treatments increased significantly (97.6-94.9 mg/dL respectively) as compared with control group(88.8 mg/dL). **Harkness and Wagner (1989)** reported the normal range of serum glucose (50-135 mg/dL) in rodents. Such differences in glucose levels could be attributed entirely to alterations in the biosynthesis of glucose due to heavy metals administration . These results are in hamony with those obtained by **Corpas *et al.*, (2002)** who concluded that the decrease in hepatic glycogen indicates that Pb is able to act on enzymes implicated in its storage and degradation. This is corroborated by the high glucose levels in blood. More over, **Calabrese and Boldwin (1992)**

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Table(4) : Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on biochemical indicators of liver function of female rat serum.

Treatments	Glucose mg/ dL	AST U/L	ALT U/L	Total bilirubin mg/ dL	Direct bilirubin mg/ dL
Group I Control	88.8* ±2.70	67.1 ±2.28	33.0 ±1.79	0.48 ±0.03	0.21 ±0.02
Group II Pb	97.6 ±2.38	96.0 ±2.56	60.2 ±3.07	0.58 ±0.03	0.27 ±0.02
Group III Pb+ Met	81.4 ±1.29	79.9 ±4.01	39.9 ±1.48	0.54 ±0.03	0.24 ±0.02
Group IV Pb+ Vit.C	85.9 ±1.03	70.50 ±2.30	40.1 ±1.59	0.50 ±0.05	0.23 ±0.02
Group V Cd	94.9 ±1.18	87.2 ±2.88	58.3 ±1.40	0.65 ±0.05	0.28 ± 0.02
Group VI Cd+ Met	80.3 ±1.72	72.7 ±2.20	47.1 ±2.30	0.62 ±0.03	0.25 ± 0.03
Group VII Cd+Vit.C	84.1 ±0.90	74.3 ±2.44	46.2 ±2.79	0.60 ±0.04	0.25 ±0.02
Mean	87.169	77.753	46.267	0.563	0.247
L.S.D 0.05	3.587	6.078	2.331	0.051	0.024

* All values are means ±SD of 7 animals.

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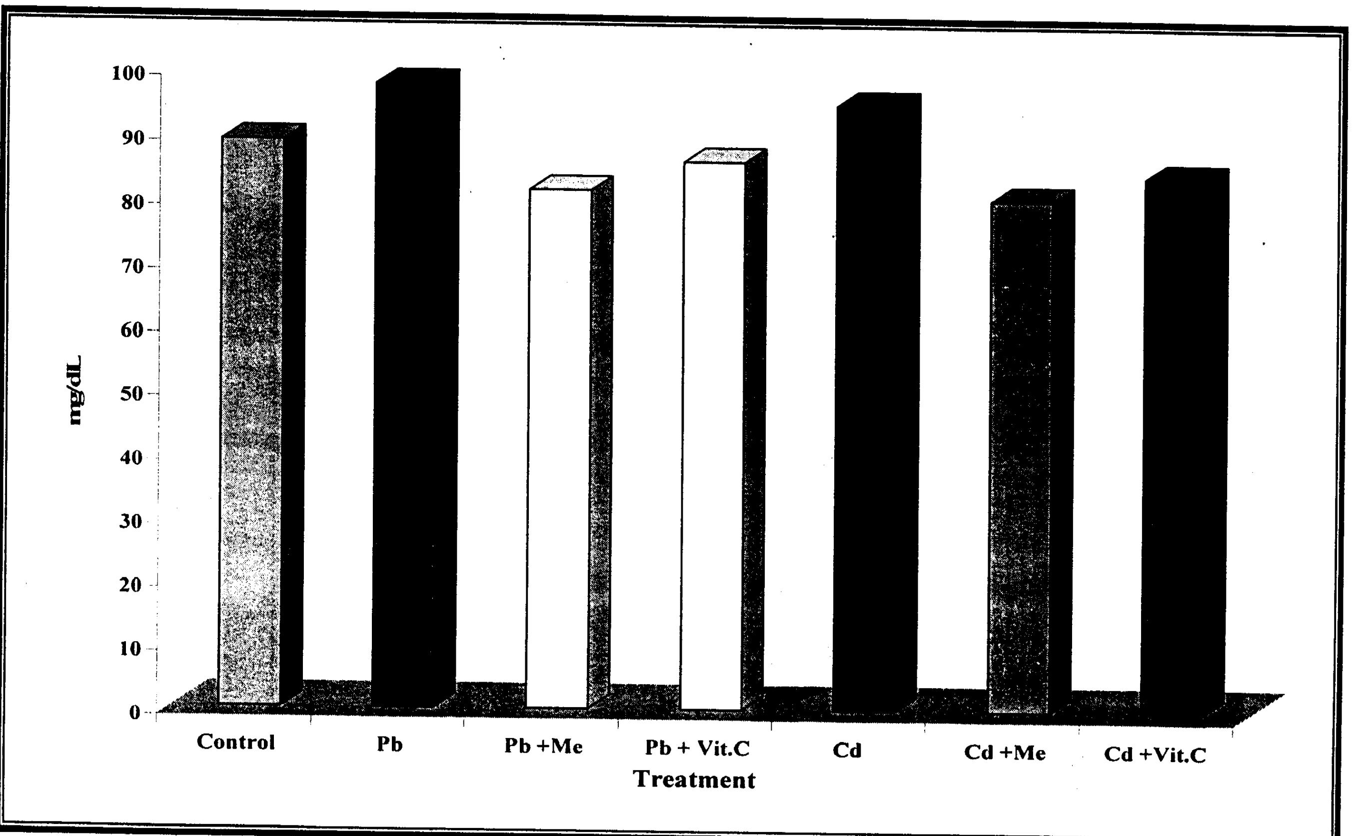

Figure (11): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on glucose in serum (mg/dL) of female rats.

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added that one the described effects of Pb exposure in experimental animals is glycosuria, because metal delays the release of insulin from the pancreas and suppressive its activity.

As regards of antioxidant effects, the obtained results showed that the administration of either methionine or ascorbic acid to Pb –groups III and IV or Cd - groups VI and VII exposed rats significantly decreased the outcome of glucose ranging from 80.3 to 85.9 mg/dL , thus bring these levels less than control (88.8 mg/dL). So antioxidant administrations appeared to provide a partial remedy against Pb –or Cd – induced oxidative stress.

In general, blood glucose may increase in diabetes mellitus and or hyper-activity of thyroid glands. Mean while, blood glucose may decrease in pancreatic disorders (Tumor), severe hepatic disorder and hypo-function of thyroid gland.

II.2.2. serum Alanine aminotransferase ALT and Aspartate aminotransferase AST:

The enzymes ALT and AST are concerned with amino acid metabolism. Large amounts of both enzymes are presenting in liver. It is well known that when there is liver cell damage the serum or plasma levels of both enzymes are raised.

Data presented in table (4) and illustrated in figure (12), showed that the mean values of serum levels of AST and ALT in control group were 67.1 and 33.0 U/L respectively. On the other hand, results showed sharp and significant increases in both enzyme due to Pb or Cd administration. It reached 43.0 and 29.9% for AST and 82.4 and 76.66 %for ALT, respectively as compared to control levels .In general, it is usually assumed that the reason for the increase of enzyme activity in serum is mainly due to the damage to the cells of liver. Such results may confirm that the inhibitory effects of heavy

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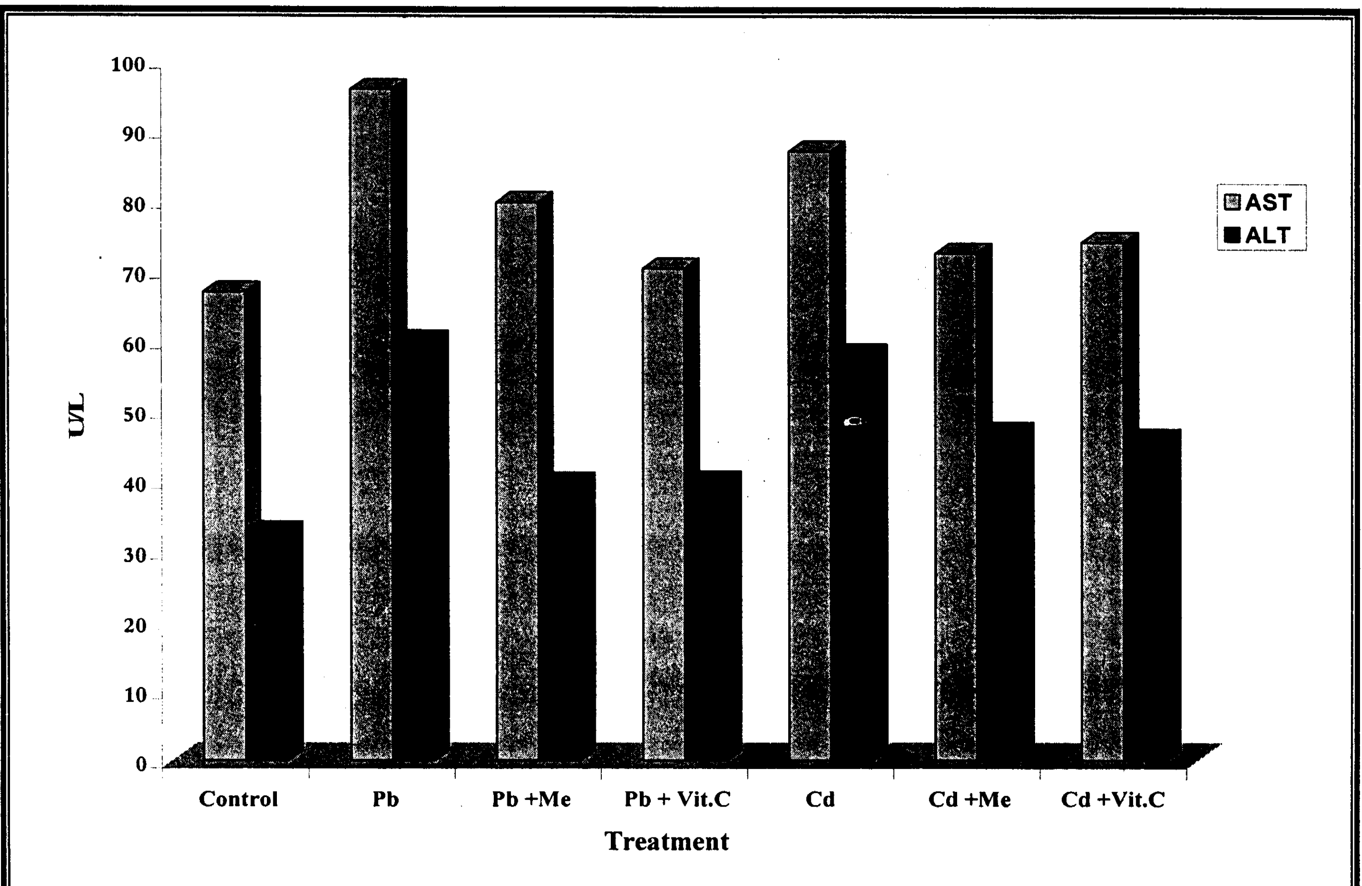

Figure (12): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on aminotransferase AST and ALT in serum (U/L) of female rats

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metals i.e. Pb or Cd on these enzymes would probably result in impaired antioxidant defense by cells and would render them more prone to oxidant attack **(Sivaprasad *et.al.* 2004)**.

The obtained results showed also that administration of antioxidant i.e., methionine or ascorbic acid along individually with Pb or Cd significantly reduced the extent of liver cell damage , resulting in lower levels of both AST and ALT as compared with Pb – or Cd – exposed rats . These results are in good accordance with the finding of **Nandi *et al.*, (2005)** who reported that methionine or ascorbic acid increases the resistances of hepatocytes to oxidation, activates liver enzymes (AST, ALT and lactate dehydrogenase) and lower the reduced glutathione in the liver. Several reports pointed out that antioxidants protect cell from oxidative damage and play vital role on detoxification of xenobiotics **Swarup and Upadhyaya, (1991); Reed and Orrenius, (1997); Guberlay *et al.*, (1998) and Ramanthan *et al.*, (2002).**

II.2.3. Total bilirubin and direct bilirubin in serum:

Bilirubin is formed from the breakdown of erythrocytes and other heam – containing proteins such as myoglobin and cytochromes. The heam (iron porphyrin) of the hemoglobin molecule is separated from the globin, and the porphyrin is converted to bilivedrin which is reduced to bilirubin. This bilirubin is referred to as indirect bilirubin. It is not soluble in water and can not be excreted in urine. It is bound to albumin and transported in blood to the liver. In liver cells, the enzyme glucuronosyl transferase joins glucuronic acid to bilirubin forming bilirubin glucuronides (mainly diglucuronides). This measurement of serum bilirubin is usually to investigate the cause of liver disease or toxicity.

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Data presented in table (4) and illustrated in figure (13) indicated that administering lead acetate or cadmium chloride to rats in drinking water resulted in statistically significant increases of both total bilirubin and direct bilirubin concentration in blood serum in relation to control group. In this concern , **Ercal *et al.*, (2001)** proposed mechanisms for Pb – induced oxidative stress in four groups: 1) Direct effect of lead on cell membranes. 2) Lead hemoglobin interaction. 3) δ- aminolevulinic acid (δ-ALA) – induced generation of ROS , and 4) Effect of lead on the antioxidant defense systems of cells. On the other hand , the same authors proposed mechanisms for Cd – induced oxidative stress can be observed in three groups 1) Adverse effects of Cd on cellular defense systems and thiol status, 2)Enhancement of lipid peroxidation by Cd , and 3) Deleterious effect of Cd on cellular enzymes .

Results also showed that adding antioxidants i.e. methionine or ascorbic acid to the diets of Pb or Cd exposed rats did not normalize such two parameters as compared to control group. Similar results were observed by **Kowalczyk *et al.*, (2003)** reporting that Cd administration to rats resulted in significant increase of total bilirubin concentration in rats exposed to Cd. **Sarkar *et al.*, (1998)** found that the increase of total bilirubin concentration in plasma after intoxication with Cd correlates with oxidative damage of other organs resulting from oxidative stress .

In the present study, it is observed that administration of methionine or ascorbic acid resulted in lowering of bilirubin concentration in serum caused by Pb or Cd treatment, referring to the beneficial change resulted from the antioxidative activity of such two antioxidants (**Flora and Tandon, 1986; Pavlovic *et.al*, 2001; Patra *et al.*, 2001; Patra and Swarup , 2004).** On the other hand **Hashim and Weshahy (2002)** and **Sadek (2004)** pointed out to the protective role of ascorbic acid against hemoglobin degradation and consequently decreased the level of serum total bilirubin in rats kept on ascorbic acid supplemented diet.

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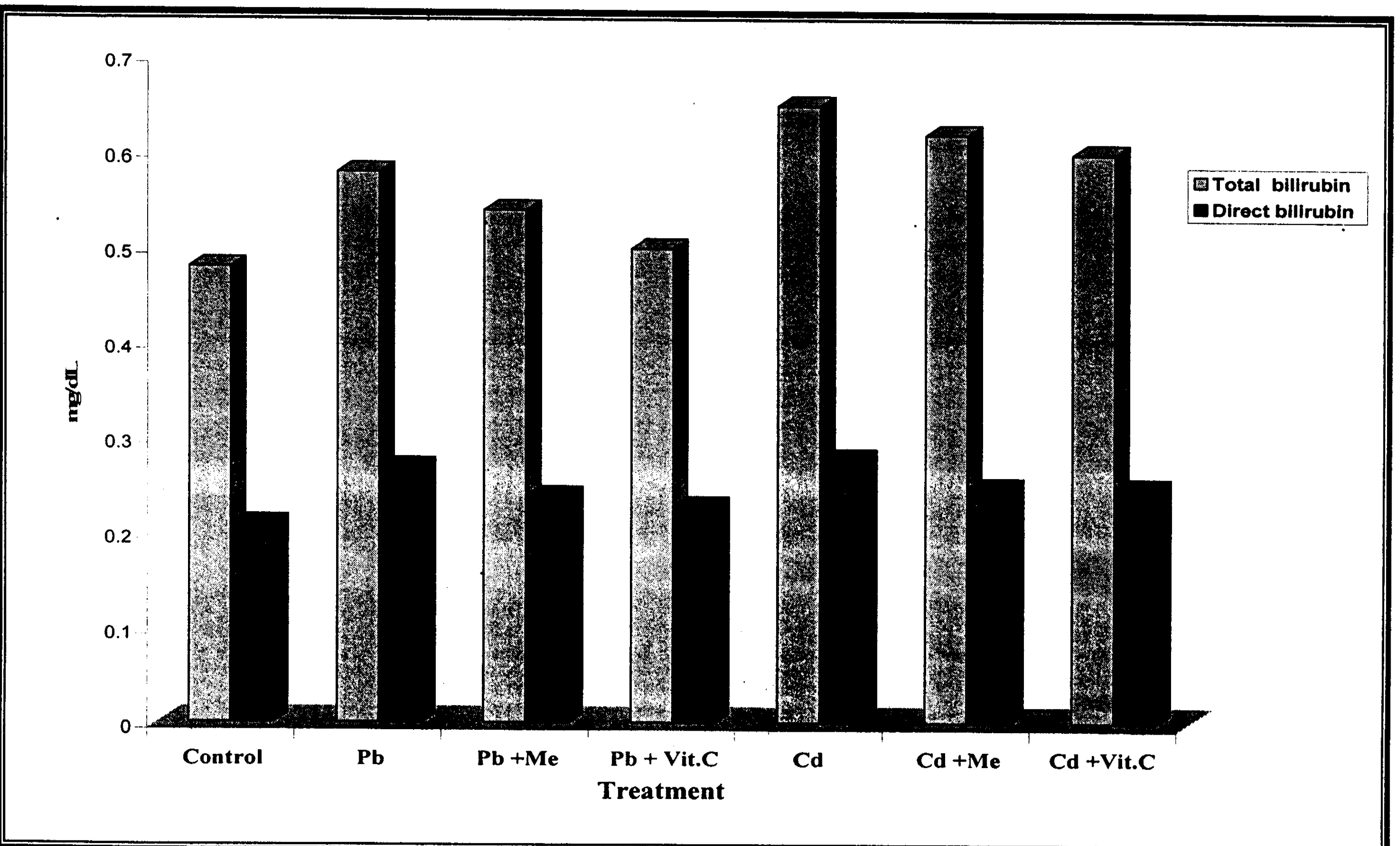

Figure (13): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on bilirubin in serum (mg/dL) of female rats.

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II. 3. Lipid profile:

Data presented in table (5) show the effects of antioxidants (methionine and ascorbic acid) and heavy metals (Pb and Cd) on lipid profile (total cholesterol, triglycerides, HDL – cholesterol and LDL- cholesterol) in serum of female rats.

As shown in Table (5) and Figures (14, 15 and 16), results showed that Pb or Cd intoxicated groups (II and V) had significantly ($P < 0.05$) high level of serum total cholesterol, triglycerides and LDL- cholesterol. Increasing of these parameters may be due to that Pb or Cd promotes cholesterol and triglycerides synthesis. However the levels of HDL – cholesterol, which is considered a major function to transfer cholesterol from peripheral tissue to liver, appeared to be low as compared to control group (I). Results also showed that Pb or Cd administrations in drinking water increased total cholesterol by 10.7 and 10.8 %, triglycerides by 56.2 and 67.8 % and LDL – cholesterol by 11.4 and 15.0 % respectively as compared to control group (I). The increase of total cholesterol in serum may be due to more than one reason: 1- increase of its synthesis or decrease of cholesterol degradation, 2-decrease of bile salts or reabsorption of bile salts is very high, and finally 3- cholesterol could not enter or penetrate the cells. However the high serum triglycerides levels intoxicated rats may be due to inhibition of lipase lipoprotein **(Iskoznynska *et al.,* 1993)**. In this concern, **Stohs *et al.* , (2001)** concluded that intake of Cd increased lipid peroxidation and resulted in change in entering a cellular stability, DNA damage and apoptosis. **Ercal *et al.,* (2001)** showed that Cd may cause adverse effects on cellular defense systems or enhance lipid peroxidation or may cause deleterious effect on cellular enzymes. In addition, **Knowles and Dolaldson (1990)** pointed out that Pb has an effect on de novo lipid synthesis in animals according to the range of dose. In general, differences in lipid profile could be attributed

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Table (5): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on lipid profile in serum of female rat serum.

Treatments	Total cholesterol mg/dL	Triglycerides mg/dL	HDL - cholesterol mg/dL	LDL- cholesterol mg/dL	HDL - cholesterol / Total cholesterol ratio	LDL- cholesterol / HDL - cholesterol ratio
Group I Control	84.5* ±1.81	99.5 ± 2.48	48.0 ±3.36	16.6 ±2.05	0.56	0.34
Group II Pb	93.6 ±4.40	155.5 ±4.16	44.0 ±2.96	18.5 ±1.65	0.47	0.42
Group III Pb+ Met	88.7±3.74	122.0 ±3.28	47.0 ±3.23	17.3 ±1.20	0.52	0.36
Group IV Pb+ Vit.C	88.4 ± 3.60	122.0 ±2.79	46.0 ±2.13	18.0 ±1.78	0.52	0.39
Group V Cd	93.7 ±3.10	167.0 ±3.29	41.2 ±3.05	19.1 ±1.33	0.43	0.46
Group VI Cd+ Met	90.0 ±2.39	137.5 ±3.03	44.4 ±2.12	18.1 ±1.25	0.49	0.40
Group VII Cd+Vit.C	87.0 ±3.30	120.0 ±2.90	45.7 ±3.14	17.3±1.67	0.52	0.37
Mean	89.247	131.92	45.18	17.84	-	-
L.S.D 0.05	3.287	3.425	3.201	1.837	-	-

* All values are means ±SD of 7 animals.

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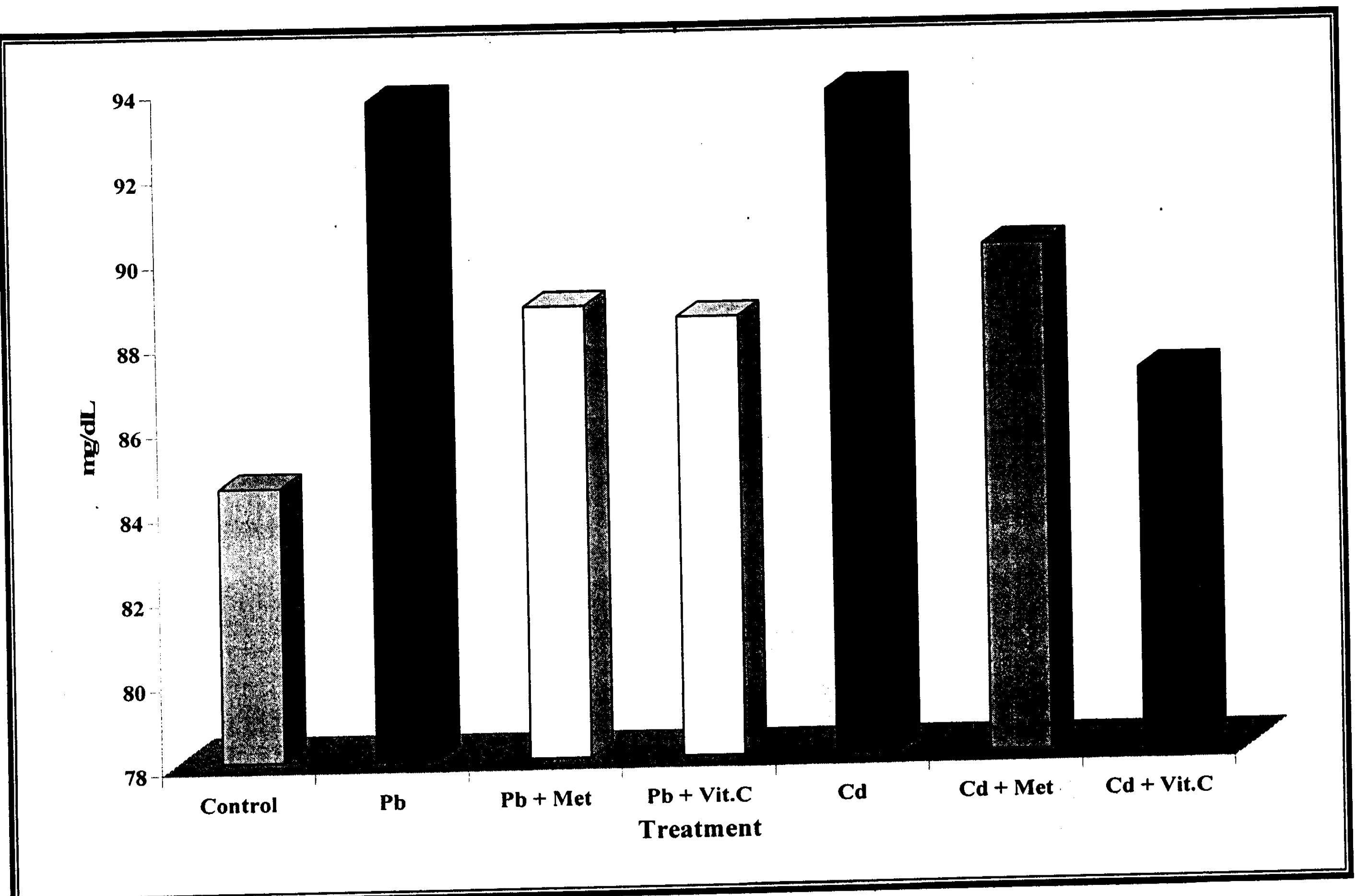

Figure (14): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on total cholesterol in serum (mg/dL) of female rats.

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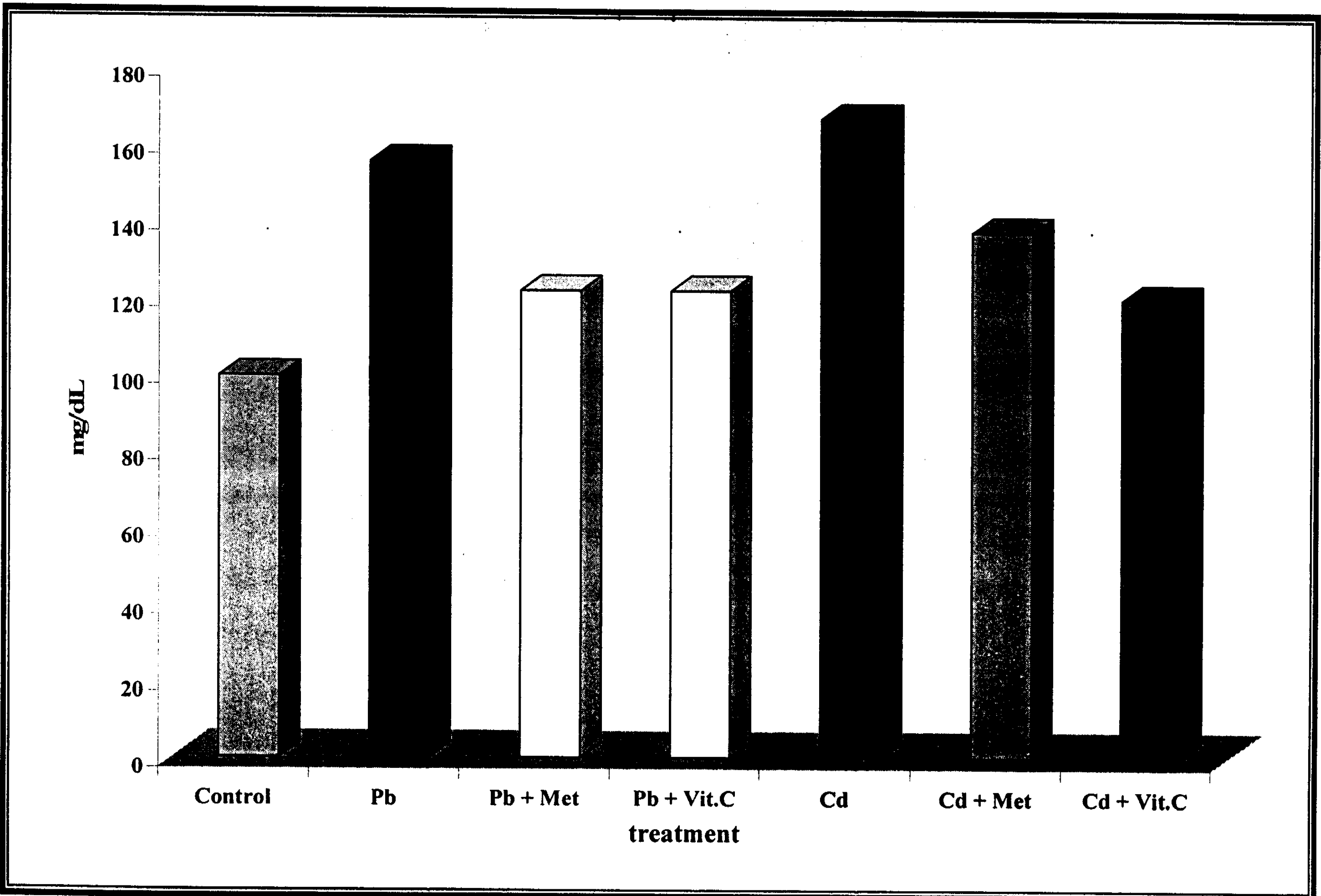

Figure (15): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on triglycerides in serum (mg/dL) of female rats.

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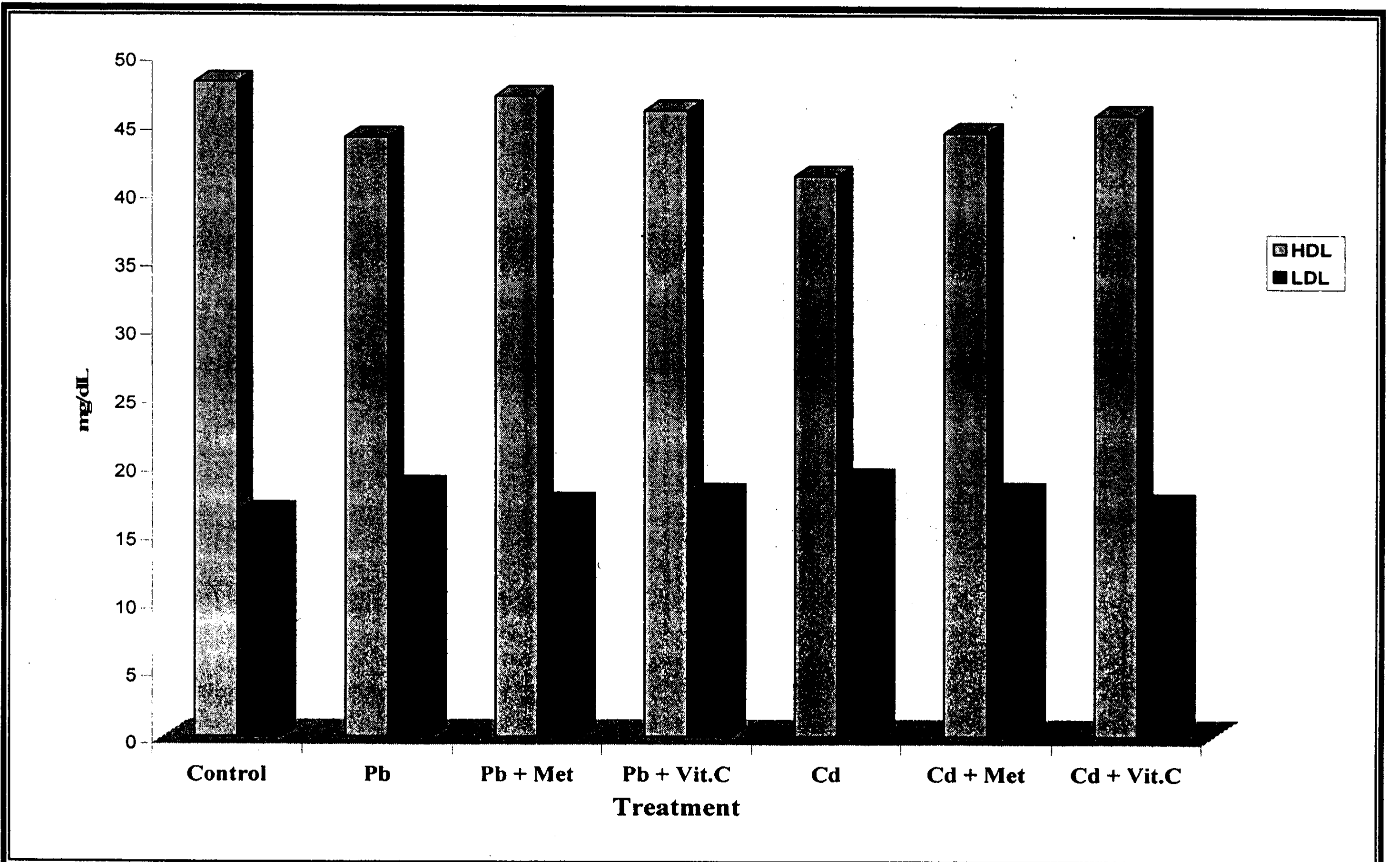

Figure (16): Effect of antioxidants (Methionine and Ascorbic acid) and heavy metals (Pb and Cd) on HDL - cholesterol and LDL - cholesterol in serum (mg/dL) of female rats.

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entirely to alterations in the biosynthesis due to metal administration. **Bertin and Averbeck (2006)** concluded that cadmium gives rise to oxidative damage affecting DNA, proteins and membrane lipids. The induction of oxidative damage is associated with mitochondrial dysfunction, deregulation of intracellular antioxidants and apoptosis. The inhibition of some DNA repair pathways contributes to the rise in mutations and cancer.

On the other hand, the significant ameliorative efficacy of both antioxidants i.e. methionine or vitamin C in experimental Pb or Cd toxicity in rats when given simultaneously were observed in the present study as compared to the Pb –or Cd – treated animals without antioxidants . Results showed lower values for total cholesterol, triglycerides and HDL –cholesterol, through not to a significant degree when compared to control group (I).

It is well known that HDL- cholesterol is good parameter for estimating the favorable effect of any substance introduced to humans or animals. Increasing HDL – cholesterol is desired due to its positive characters.

Results showed also that antioxidants have the effect of improving blood lipid status via increasing HDL – formation and cholesterol excretion. The newly formed HDLs are nearly devoid of any cholesterol or cholesterol ester.

Holloway and Rivers (1981) reported that Vit.C stimulates the activity of cholesterol 7-alph hydroxylase enzymes, which regulate the conversion of cholesterol to bile acid a vehicle for cholesterol excretion. Also, **Nagyova *et.al.* (1994)** found that high L-ascorbic acid (100gm daily) intake of 12 weeks, significantly decreased serum, liver and aorta cholesterol concentrations in cadmium treated guinea pigs. **Joyachandran *et.al.* (1996) and Nambisan and Kurup (1974) reported** that ascorbic acid administration brought down the levels of cholesterol and TGs in aged and young rats. Also, the present results showed that antioxidants seemed to raise the HDL- cholesterol / total cholesterol ratio (0.52, 0.52 , 0.49 and 0.52 for groups III , IV, VI and VII respectively) as compared to intoxicated treatments (0.43- 0.47 for group V and II , respectively

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). However , The positive correlation between serum HDL- cholesterol and ascorbic acid application was stated by **Sharma *et al.*, (1988), Judith *et al.*, (1994), Ness *et al.*, (1996), Farag *et al.*, (2003) and Sadek (2004).**

Such results are in accordance with these reported for the ameliorative effect of ascorbic acid in experimental Pb toxicity **Flora and Tandon, (1986),** phosphine – induced oxidative damage in rats **Hus *et al.*, (2000)** and in acute arsenic toxicity of rats **Ramanathan *et al.*, (2002).** Also **Sadek (2004)** showed that supplementation diet with 300mg ascorbic acid /kg diet resulted in favorable diminishing effect on serum total lipids, and exerted significant lowering effect on serum total cholesterol.

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SUMMARY

The aim of this study was to assess the protective effects of natural antioxidant (DL- methionine and L- ascorbic acid) on lead or cadmium toxicity where they tested separately for their effects on selected indices of changes in body weight and weight of some organs of female rats, as well as some blood serum biochemical parameters.

Female albino rats of *Winstar* strain were used. A total number of 49 rats were divided into seven groups.

A dose of 300 ppm of lead as lead acetate and 40mg cadmium as cadmium chloride were administered through drinking water for 5 weeks. Rats received methionine – diet (6.5g/kg diet) and ascorbic acid- diet (250 mg / kg diet). On the completion of 5 weeks, animals were scarified at the end of experiment, liver, kidneys, lung, and heart were excised immediately and weighed. Blood samples were collected and centrifuged at 3000 rpm to obtain serum.

The obtained results could be summarized as follows:

1-Body weight

Exposure of Pb or Cd reduced significantly the weekly body weight of rats. However, the beneficial role of antioxidant with heavy metal treatment was observed where the relative weight gain % was increased.

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2- Organs weight :-

All rat organs weight were significantly affected by Pb or Cd intoxication. Liver and kidneys were target organs for heavy metals accumulation. However, supplementation of antioxidants in diets reduced liver and kidneys weights of exposed rats.

3- Renal functions:

Heavy metals lowered serum total protein , methionine treatment with Pb toxicity could not increase it , while ascorbic acid administration showed more efficient in both exposed – Pb or -Cd rats where it increased . Pb or Cd exposure increased significantly the concentration of urea and creatinine in serum, methionine and Vit.C reduced such two parameters in exposed animals.

4-Liver functions:

Administration of Pb or Cd increased significantly blood serum glucose; however, antioxidants decreased the outcome of glucose and brought its level less than control.

Sharp and significant increases in both aminotransferases enzymes i.e ALT and AST were observed in blood serum of exposed rats . Antioxidants i.e methionine or ascorbic acid along individually with Pb or Cd administration significantly reduced the extent of liver cell damage (lowered the levels ALT and AST).

There were significant increases in total bilirubin and direct bilirubin concentration in blood serum after intoxification with Pb

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or Cd . However , adding methionine or ascorbic acid to the diets of exposed rats did not normalize such two parameters as compared to control group.

5- Lipid profile:

Lead or cadmium intoxicated groups contained significant high levels of serum total cholesterol , triglycerides and low density lipoprotein –cholesterol (LDL –cholesterol) , however the levels of HDL- Cholesterol appeared to be low as compared to control group .

The ameliorative efficacy of both antioxidants in experimental Pb or Cd toxicity were observed . It decreased the values for total cholesterol , triglycerides and LDL – cholesterol and improved blood lipid status via increasing HDL formation and cholesterol formation .

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ARABIC SUMMARY

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دراسات كيمائية حيوية علي بعض مضادات الأكسدة الطبيعية

أجريت هذه الدراسة بهدف تقييم دور بعض مضادات الأكسدة الطبيعية مثل حمض الميثونين وحمض الاسكوربيك لتقليل الأثر السام وحماية فئران التجارب المعرضة للتسمم بالعناصر الثقيلة مثل الرصاص والكاد ميوم وذلك عن طريق بعض القياسات مثل الزيادة في وزن الجسم الاسبوعي ووزن بعض أعضاء الفئران الداخلية مثل الكبد والكليتين والطحال والقلب والرئتين بالإضافة إلي بعض المكونات البيوكيميائيه لسيرم الدم.

استخدم في هذة الدراسه فئران تجارب سلالة *Winstar* بعدد 49 فأر أنثي قسمت ألي سبعة مجاميع ولمده خمسة أسابيع متتالية وتناول فيها الفئران وجبات مدعمه بحمض الميثونين (6.5 جرام / كيلو وجبه) أو حمض الاسكوربيك (250مللجرام/ كجم وجبه) وكان ماء الشرب مضاف إليها العناصر الثقيلة (300 جزء في المليون من الرصاص علي صورة خلات الرصاص و 40 مللجرام كادميوم علي صورة كلوريد الكادميوم .

وفي نهاية مدة التجربة تم ذبح الفئران وتم استئصال الاعضاء منها ووزنها في الحال وتم الحصول علي سيرم الدم بعد عملية طرد مركز علي 3000 لفه / دقيقة .

وكانت اهم النتائج المتحصل عليها كما يلي :-

1- **وزن الجسم :**

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الفئران التي تعرضت للملوثات مثل Cd او Pb ادي ألي نقص معنوي في وزن الفئران اسبوعياً وعند اضافة مضادات الاكسدة كان لها تأثير مفيد حيث ادت ألي زيادة النسبه المئويه لمعدل الزيادة في الوزن للفئران المعرضة للتلوث.

2- وزن الاعضاء :-

تأثرت معنويا أوزان كل الأعضاء المفصولة من الفئران المعرضة للتلوث . وكانت الكبد والكليتين مركزا لتراكم عناصر الرصاص والكادميوم بهما ووضحت الزيادة الكبيرة في أوزانهم . وعند إضافة مضادات الأكسدة نقصت أوزانهما في الفئران التي تعرضت للتسمم .

3- وظائف الكليتين :

أدي التلوث بالعناصر الثقيلة ألي نقص لكمية البروتين الكلي فى سيرم الدم في الفئران المعرضة للتلوث .وعند اضافة الميثونين ألي الوجبه لم يكن هناك زيادة في كمية البروتين الكلي بينما ادي اضافه حمض الاسكوربيك ألي الوجبة ألي زيادة هذا المكون في سيرم دم الفئران المعرضة للتلوث . وعموما أدي تلوث مياه الشرب بالعناصر الثقيلة إلي زيادة معنوية لكل من اليوريا والكرياتينين في سيرم دم الفئران المعرضة للتسمم ولكن عند اضافة مضادات الاكسدة في الوجبات المقدمة لهذه الفئران قللت معنويا كل من اليوريا والكرياتينين في سيرم الدم .

4- وظائف الكبد :

اضافة الرصاص او الكادميوم لماء الشرب للفئران ادي إلي زيادة معنويه لكمية الجلوكوز في سيرم الدم وعند اضافة مضادات الاكسدة في وجبات الفئران قلل

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كمية الجلوكوز في سيرم الدم باقل من كميتها في سيرم دم مجموعة فئران المجموعة الضابطة (الكونترول).

كانت هناك زيادة حادة ومعنوية في وحدات الانزيمات الناقلة لمجموعة الامين AST و ALT نتيجة التلف الحادث للكبد بتأثير العناصر الثقيلة الموجوده في مياه الشرب وكان هناك تأثير مفيد لمضادات الاكسدة عند اضافتها للوجبات حيث قللت ألي حد كبير من تلف الكبد وكان واضحاً بنقص نشاط كل من AST و ALT .

وكانت هناك زياده كبيرة ومعنوية لكل من Total bilirubin and direct bilirubin في سيرم دم الفئران المعرضة للتلوث ولكن عند اضافة مضادات الاكسدة في وجبات الفئران المعرضة للتلوث لم يصل مستويات هذين المكونين إلي مستوي المجموعة الضابطه (الكونترول) .

5- أشكال الدهون:-

اظهرت مجموعات الفئران المعرضة للتلوث مستوي عالي ومعنوي لكل من الكوليسترول والجلسريدات الثلاثية وكوليسترول البروتينات ذات الكثافة المنخفضة – والضار LDL ونقص الكوليسترول البروتينات ذات الكثافة العالية - HDL المفيد في سيرم الدم عند مقارنتها بمجموعه الكونترول وكان لإضافة مضادات الأكسدة لوجبات الفئران المعرضة للتلوث تأثيرا مفيدا حيث قلل من كميات المكونات الزائدة نتيجة التلوث وادي إلي إفادة أكثر بزيادة كمية كوليسترول البروتينات عالية الكثافة المفيدة للجسم .

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