CHAPTER - 1

REVIEW OF LITERATURE

1.1. CADMIUM

1.1.1 Introduction

Cadmium, a heavy toxic metal that is widely used in industry, affects human health through occupational and environmental exposure. It is present in soils, sediments, air and water and is listed by the US Environmental Protection Agency as one of 126 priority pollutants (Waisberg et al., 2003). It is considered one of the most toxic substances in the environment due to its wide range of organ toxicity and long elimination half-life of 10-30 years (Jarup et al., 1998). It has been estimated that at least 512,000 U.S. employees each year work in an environment that potentially exposes them to cadmium (Wittman and Hu, 2002).

1.1.2 Occurrence

Cadmium-contaminated topsoil, however, is considered the most likely mechanism for the greatest human exposure through uptake into edible plants and tobacco (ATSDR, 1999). The EPA estimates approximately 3.4 billion pounds of sewage sludge are transferred to soil annually in the United States (ATSDR, 1999). Today, the main uses for this metal are for nickel-cadmium battery manufacture, pigments and plastic stabilizers. Anthropogenic sources of cadmium in the environment are from refining, copper and nickel smelting, and fossil fuel combustion. Natural sources of cadmium in the atmosphere are from volcanic activity, forest fires and wind-borne transport of soil particles. Irwin et al. (2003) reported that anthropogenic sources add 3-10 times more cadmium to the atmosphere than natural sources. Major occupational exposure occurs in non-ferrous smelters, from the production and processing of cadmium, its alloys and compounds, and increasingly in the recycling of electronic waste. Non occupational exposure is mainly from cigarette smoke that contains high concentration of cadmium. For non-smokers who are not occupationally exposed, diet is the main source of exposure to cadmium.

Generally, cadmium is always found in association with around 5 percent levels of zinc. It has been estimated that approximately 500 tons of cadmium enters the environment annually as a result of natural weathering and about 2000 tons as a result of human activities. About 20 percent of released cadmium comes from zinc mining and smelting operations with another 30 percent from the manufacturing, use and disposal of cadmium products. The

remaining 50 percent is dispersed as a contamination in other substances, including phosphate fertilizers, sewage effluent and sludge. Moreover, it is also released during combustion of fossil fuels. Fertilizer raw materials are also contaminated with cadmium; 1.3 million pounds of cadmium-contaminated zinc sulphate containing up to 215,000 ppm cadmium entered the United States in November 1999 alone. It is not known how much of this product has been sold and applied to agricultural lands (Washington State Department of Ecology, 2000). Fertilizers continue to be contaminated with cadmium as a result of the recycling of industrial waste sold as zinc sulphate or other raw materials for agricultural and home use fertilizers. Assays of commonly sold dry fertilizer and soil amendment in Washington State in 1998 revealed concentrations of cadmium as high as 160 mg/kg dry weight (Washington State Department of Ecology, 1999). Cadmium is distributed in the different components of the environment as follows:

1.1.2.1 Cadmium In Air

The ambient air that we breathe comes from the lower atmosphere (below 80 km). Pollutants such as dust, smoke, soot, industrial and automobile exhaust and gaseous and particulate matter, are found in varying concentrations depending upon factors, such as population, vehicular density, location and type of industrial units in that area.

Data on the concentration of cadmium in air of United States collected by National Air Sampling Network have been summarized by Tabor and Warren (1958), and by Schroeder (1970). The atmospheric concentration range of cadmium in different parts of the world such as Greenland is 0.003-0.63ng of Cd/m^3, North America 21ng of Cd/m^3. In the same study it is reported that in North India the concentration of these metal is 330-2100ng of Cd/m^3. Data was collected in 1966 (Schroeder, 1970) for 58 cities and 29 non-urban areas giving range of concentration of cadmium (ng/m^3): 2-370 for urban areas and 0.4-26 for non-urban areas. Friberg et al. (1971) quoted weekly means of cadmium $500ng/m^3$ at a distance of 100 meters and $200ng/m^3$ at a distance of 400 meters from a Japanese smelter. Cadmium occurs mostly in the particulate phase. Particles of 0.1-10μm in diameter settle very slowly and remain suspended in the air for a longer duration of time. The typically small size (especially the particles<1μm in diameter) of these particulates allows them to be inhaled deeply into the respiratory tract. Metal ions are absorbed by the lungs ten times more than the intestine.

The World Health Organization (WHO) guideline for the maximum permissible concentration of cadmium in air is 10-20ng/m^3/yr.

1.1.2.2 Cadmium In Soil

The metal toxin cadmium (Cd) is found in low abundance in the earth crust and most surface soils (international Programme on Chemical safety, 1992; Waalkes et al., 1992; Jarup et al., 1998). High concentrations of Cd can be found in soils in an area rich in zinc ores in which the toxin Cd occurs naturally in high abundance together with lead and copper (Jarup, 2003). This makes Cd a by-product or a waste product of industrial production of zinc, lead and copper. Increasing levels of Cd in agricultural soils is attributable to the use of Cd-containing phosphatic fertilizers and environmental pollution associated with industrial zinc production activities (Jarup, 2003). Cd exhibits higher rates of soil to plant transference than do other metal toxins such as lead and mercury. In consequence, it enters the food chain and it is present in most human food stuffs albeit in varying quantities (Galal-Gorchev, 1993; Australia New Zealand Food Authority, 1998; Kikuchi et al., 2002).Analysis of uncontaminated soils indicates that the normal content of cadmium is about 0.4ppm of cadmium on the average (Fleischer et al., 1974). The data of Langerwerff (1971) clearly shows the extent of soil contamination near highways. Contamination from cadmium occurs in various other natural materials such as magnetic rocks (0.026-0.130 ppm), shales (0.300 ppm), limestones (0.035-0.09 ppm) and sediments (0.39-0.57 ppm). The contents are significantly higher in the industrial and airport zone than in the residential areas.

1.1.2.3 Cadmium In Water

Most fresh waters contain less than 1 ppm of cadmium. The surface water that contains more than a few ppm cadmium near urban areas have almost certainly been contaminated by industrial wastes from metallurgical plants, plating works, or plants manufacturing cadmium pigments, cadmium stabilized plastics or nickel-cadmium batteries or by effluent from sewage treatment. Soni (1990) has reported that the industrial effluent of dye and pigments contains 0.004-0.009 ppm cadmium, whereas chemical industry effluent contains 0.015-0.075 ppm cadmium. The buildup of cadmium into water, and sediments in higher than

natural levels results in bioaccumulation of the metal in various pockets of the food chain. The concentration of cadmium in plant food is given in table 1.1.

The uptake of cadmium from the soil through produce results in elevated concentration in vegetables, fruits and grains, with the highest levels in leafy greens and potatoes. High levels are also found in shellfish (up to 30mg/kg) and organ meats.

Large quantities of Cd are found in offal notably liver and kidney and in certain bivalve mollusks and crustaceans because of their ability to concentrate Cd from aquatic environment (Kikuchi et al., 2002; Prankel et al., 2004).

Table 1.1. Cadmium in common plant food

PLANT FOOD	CONCENTRATION (mg/kg food)
Cereals (wheat, rye, rice)	0.016-0.75
Potatoes	0.03-0.05
Vegetables(lettuce,cabbage,spinach,celery,cucumber)	0.04-0.067
Fruits (peaches, bananas, apple, citrus fruits)	0.008-0.011
Mushrooms	0.46
Tea	0.21
Coffee	0.17

1.1.3 Toxicokinetics

Ingested cadmium is poorly absorbed. Approximately 5% of the total cadmium ingested in food or water is absorbed. Cadmium absorption increases with iron or calcium deficiency. Absorption from the gut appears to take place in two phases- uptake from the lumen into the mucosa, and transfer from the mucosa into the circulation. Cadmium is distributed throughout the body, but the major portion is found in the liver and kidney. The majority of absorbed cadmium is retained in the tissues. Half-times for cadmium in the human kidney have been estimated at 6-38 years and in human liver at 4-19 years. Cadmium concentration

in the kidney is near zero at birth, but rise with age to a peak (generally around 40-50μg Cd/g wet weight) between ages 50 and 60, after which renal concentration plateau or decline. Hepatic concentrations of cadmium also are near zero at birth, increasing to values of 1-2μg Cd/g wet weight by age 20-25, and increase only slightly thereafter. Thus, renal concentrations far exceed hepatic concentrations following prolonged exposure. Cadmium does not undergo metabolic conversion, but the cadmium ion can readily bind to anionic groups, especially sulfydryl groups, in proteins and other molecules. Cadmium is bound to the protein metallothionein in the liver, which releases the metallothionein-cadmium complex, rather than free cadmium, into the blood stream. Metallothionein is a low-molecular-weight, sulfhydryl-rich protein that normally binds zinc. Metallothionein-bound cadmium is readily filtered by the renal glomerulus and reabsorbed from the glomerular filtrate by the proximal tubule cells, at which point the "exogenous" metallothionein is catabolized in tubular lysosomes, releasing free cadmium. The free cadmium stimulates the synthesis of metallothionein in the tubular cells, is then bound to the tubular metallothionein, and remains in the cells. Most of the ingested cadmium is excreted unabsorbed in the feces. Most of the absorbed cadmium is retained; some excretion of cadmium occurs through the urine, and urinary excretion increases with renal damage (ATSDR, 1999a)

1.2 NORMAL HUMAN INTAKE

Cadmium is found more in food than air and water. The current federal minimal risk level (MRL) for cadmium – a level at which chronic exposure in humans is not likely to cause cancer or adverse health effects- is 0.2 μg/kg/day (14.0 μg the average adult). The average American diet in 1986 provided 0.4 μg/kg/day of cadmium (ATSDR, 1999). The overall range of dietary cadmium in Swedish diets in 1994-1996 was 2-175 μg/day and is estimated to be increasing at a rate of two percent per year (Jarup et al., 1998). The World Health Organization has shown that dietary cadmium exposure has a very wide range: inhabitants of worldwide non polluted areas have a daily dietary intake of approximately 40-100 μg, while inhabitants of polluted areas may obtain 200 μg or more as an average daily intake (Jarup et al., 1998). But in smokers Lewis et al. (1972a) reported it as an important source of cadmium. Autopsies were performed on 172 adults, including 45 male smokers whose approximate cigarette consumption was known, and 23 non-smoking males. The mean age at

death for each group was 60 years. Cadmium levels in lungs, liver, and kidney were determined by using atomic absorption. The estimated body burden of cadmium in non-smokers averaged 6.63 mg and was double the amount, 15.8mg in the smokers. Cd is an integral constituent of tobacco because of the propensity of the Nicotiana species to concentrate Cd independent of soil-Cd content. Tobacco Cd content varies widely, but a typical range is 1-2μg/g dry weight, equivalent to 0.5-1 μg/cigarette (Elinder et al., 1983). Cd oxide generated during the burning of cigarettes is highly bioavailable. Approximately a 10% of the inhaled Cd oxide is deposited in lung tissues, and another 30-40% is absorbed into the blood circulation of smokers. In a study it was shown that normal Thai men who on an average smoked 9 cigarettes per day for 9 years had approximately 2-fold greater body Cd load than did non-smoking men of the same age (Satarug et al., 2004). In another study on men older than 50 years of age in northern Taiwan, smokers were 2.5 times more likely to excrete higher urinary Cd levels than nonsmokers (Chen et al., 2003). Szadkowski (1969) reported that about 1.4μg of cadmium found in a cigarette would be in particulate phase and 0.03μg in the gaseous phase. About 0.1-0.13μg might be inhaled per cigarette. The respiratory intake from two packs per day would be about 4-6μg or 10-20 times the intake from the reported levels in the air of lower Manhattan.

Cadmium occurs in small amount in all food used by man and animals. Friberg et al. (1971) have reviewed data on cadmium in food in various countries and there seems to be general agreement that food average about 0.05ppm cadmium (net weight), of course, with wide variation depending on the source. Murthy et al. (1971) reported 27-64μg/day cadmium intake via food. Further evidence of approximate correctness of the figure of 50μg/day is provided by data on daily fecal excretion of cadmium in the general population. Thus, Tsuchiya (1969) reported that daily fecal excretion in four non-occupationally exposed men was 57μg.The studies of water supplies indicate that except for unusual instances of contamination, the intake via water is probably negligible. The average intake from drinking water is about 1 or 2μg/day. Airborne particulate or aerosols provide an additional source of cadmium to the body.

In summary, it can be stated that intake for men under ordinary circumstances is principally from food, and most estimates would put this at about 20-50μg/day. Due to poor absorption

from intestinal tract it is possible that only about 2μg/day or less is actually assimilated. The intake from drinking water is presumably 1 or 2μg/day on the average due to poor absorption. The intake from ambient air is probably very low. The daily assimilation of cadmium from ambient air is approximately 0.02 μg.

1.3 DISPOSITION

Tipton and Stewart (1970) described a balance study in normal subjects over a period from 140 to 347 days. Using atomic absorption methods, the amounts of cadmium were determined in the diet, and the amount excreted in the urine and feces were measured. The subjects were reported to have ingested an average of 170 μg Cd/day from the diet and to have excreted about 42 μg/day in the feces and 94 μg/day in the urine.

Decker et al. (1958) measured cadmium levels in the kidney and liver. These contained about 0.3 to 0.5 percent of the dose of cadmium ingested in one year. Since 50 to 75 percent of the body burden will be in these organs it is clear that overall retention probably was only 1 percent or less of the dose. Friberg et al. (1971) have also reported that monkeys retained only about 3 percent of an ingested dose 10 days after ingestion. Another method of estimating absorption in humans is to determine body burden at autopsy and estimate total intake over a half-life. Though subject to many errors, such calculations are compatible with an absorption rate of about 3 to 8 percent (Friberg et al., 1971).

There is strong evidence that cigarette smoking contributes substantially to the cadmium body burden of smoker (Lewis et al., 1972a; 1972b). Rough calculations suggest that the retention of cadmium in cigarette smoking is substantial. Lewis et al. (1972a; 1972b) estimated that the body burden of cadmium due to cigarette smoking is 0.36 percent mg/pack-yr (where 1 pack-yr denotes one pack smoked per day for one year). On estimating the sum of cadmium in liver, kidney and lungs attributable to cigarette smoking as 25mg/100 pack-yr and subtracting 7mg from other sources giving 18mg. As the total body burden is assumed to be twice the burden in these organs, yielding (18mg X 2)/100 pack-yr or 360 μg/pack-yr. Menden et al. (1972) reported that approximately 2 μg cadmium is inhaled per pack implying that 1 pack-yr provides 730 μg. Cd inhaled (2 X 365 = One smoked per day for one year).

In summary between 10-50 percent of cadmium fumes are absorbed through the respiratory tract and approximately five percent of oral cadmium is absorbed through the digestive tract. Smokers absorb 1-2 µg cadmium per pack of cigarettes, approximately doubling the average exposure of a nonsmoker and doubling the average amount of cadmium found stored in the kidneys. Although absorption through the gastrointestinal tract is significantly lower, low dietary intakes of calcium, protein, zinc, iron and copper may increase cadmium absorption in the gut.

1.3.2 Cadmium Metabolism

When Cadmium is absorbed it circulates in erythrocytes or bound to albumin. In the liver it can induce and bind to metallothionein, a cysteine- rich protein that can concentrate cadmium up to 3,000-fold (Klaassen et al., 1999). The metallothionein/cadmium complex is slowly released over time from the liver and circulates to the kidneys where it can accumulate in renal tissue. Cadmium also accumulates in the bone, pancreas, adrenals and placenta. The majority of accumulation, approximately 50% of total body stores, occurs in the liver and kidney (Pope and Rail, 1995).

1.4 MECHANISM OF CADMIUM TOXICITY

The mechanisms responsible for Cadmium induced toxicity may be multifactorial. Cd has been officially listed as a pulmonary carcinogen for rats and humans by the International Agency for Research on Cancer (IARC Monograph, 1993). Proposed mechanisms of Cd-induced oxidative stress can be studied in three groups:

1.4.1 Adverse effects of Cadmium on cellular defense systems and Thiol status:

Cadmium remains in the lungs and induces a cysteine (Cys) rich protein called metallothionein (MT). There are several isoforms of MTs, which are known to protect cells from oxidative stress. Since MTs are comparatively cysteine-rich (20%-30% of the protein is Cys), and metals have a high affinity for thiols, MTs are known to sequester metals (Simpkins, 2000). Therefore, metals (particularly cadmium) are stored as Cd-MT complex in the liver. This Cd-MT is transferred from the liver to the kidneys over time, and is then filtered and reabsorbed by the renal proximal tubules. Cd-MT is metabolized in lysosomes to

liberate cadmium ions. These liberated cadmium ions again bind to preexisting or newly made MT. If MT synthesis cannot keep up with the demand and the non-MT bound cadmium overwhelms defense systems, cadmium toxicity ensues (Klaassen and Liu, 1997; Gong and Hart, 1997).

Several studies indicated that cadmium alters GSH levels. For example, cadmium administered orally for 30 days was found to increase tissue GSH levels in rats (Rana and Boora, 1992). In another study, GSH was also increased in the livers and kidneys of rats given 0.228 Cd/kg, 3days/week for 1-year (Kamiyama et al., 1995). GSH is known to protect cells against oxidative stress and any alteration in GSH levels (either a decrease or an increase) indicates a disturbed oxidant status. When cells are oxidatively challenged, GSH synthesis increases. As oxidative stress continues, GSH synthesis cannot efficiently supply the demand; therefore, GSH depletion occurs. Alterations in GSH levels have been observed in cadmium toxicity, and most studies reported an increase in GSH levels after cadmium exposure (Rana and Boora, 1992; Kamiyama et al., 1995; Rana and Verma, 1996; Shaikh et al., 1999). A few studies reported GSH depletion in tissues (Karmarkar et al., 1998; Shibasaki et al., 1996). In order to support the hypothesis that cadmium toxicity changes GSH levels, a GSH-depleting agent, buthionine sulfoximine(BSO), was given to animals (BSO inhibits γ-glutamylcysteine synthetase). Depletion of GSH caused nephrotoxicity within 6 hours after a cadmium injection as indicated by a several-fold increase in urinary lactate dehydrogenase (LDH) activity. Acute hepatotoxicity was not observed in animals treated with BSO and Cd (Gong and Hart, 1997) Moreover, co-treatment with N-acetylcysteine (NAC), a GSH replenishing agent and a free radical scavenger, protected against chronic hepatotoxicity and nephrotoxicity as well. These studies indicate that cadmium toxicity causes oxidative stress by challenging the thiol status of cells.

1.4.2 Enhancement of lipid peroxidation by Cadmium:

Lipid peroxidation has also been observed in cadmium toxicity. Malondialdehyde (MDA) is a well known lipid peroxidation indicator and has been found to increase in the liver and kidneys after cadmium exposure (Shaikh et al., 1999). Reasons for lipid peroxidation after cadmium exposure are not completely known, but we believe that disturbances in GSH and MT levels may allow free radicals to be "free" such that OH and O_2 radicals can attack

double bonds in membrane lipids and result in an increase in lipid peroxidation. Moreover, mitochondrial respiration as the major source of ROS is promoted by lipid peroxidation and therefore enhances oxidative stress induced cadmium toxicity (Karmarkar et al., 1998). In another study, Yiin et al. demonstrated that administration of cadmium in various doses significantly increased thiobarbituric acid-reactive substances (TBARS), a well-known indicator of lipid peroxidation, in rat adrenal glands that structurally contain large amounts of polyunsaturated lipids (Yiin et al.,2001). This same research group also showed the presence of lipid peroxidation of arachidonic acid catalyzed by cadmium chloride in vitro. In this particular study, it was indicated that when cadmium concentration, exposure time and temperature of incubation were increased, the production of lipid peroxidation was also elevated (Yiin, 1998).

1.4.3 Deleterious effects of Cadmium on cellular enzymes:

Metal-induced alterations in antioxidant enzyme activities have been extensively studied over the years. It has been reported that especially Catalase (CAT) and Superoxide dismutase (SOD), two major antioxidant enzymes are affected by cadmium. SOD activity was found to be inhibited by administration of cadmium acetate to liver and kidneys *in vitro*, as well as *in vivo*. High levels of lipid peroxidation were found in both tissues (Hussain et al., 1987). Mateo et al. (1997) examined CAT activity in erythrocytes. Administration of 0.2mM Cd inhibited CAT activity in colon cancer and showed less malignancy whereas it increased CAT activity in gastric neoplasia (Shaikh et al., 1999). Aminotriazole (AT) is a CAT inhibitor. Shaikh et al.(1999) studied the effects of AT on cadmium nephrotoxicity. A single dose of AT was given to rats, prior to the last cadmium injection. A significant increase in urinary LDH activity was seen indicating nephrotoxicity (Mateo et al., 1997).

Some of the cellular effects of Cadmium are known. Fifty to sixty percent of exposed populations have been shown to have chromosomal damage (Fowler, 1978). Cadmium is known to bind to the mitochondria of the cell and is capable of inhibiting both cellular respiration (by 75%) and oxidative phosphorylation (by 100%) at low concentrations. This mitochondrial toxicity can completely inhibit the hydroxylation of vitamin D in renal tissue at concentrations of 0.025 mmol (Nordberg et al., 1985).

Some of the specific changes that lead to tissue damage and death in chronic exposure have been related to oxidative stress and thiol depletion (Ercal et al., 2001). Cellular damage results from cadmium binding to sulfhydryl groups in tissue, the production of lipid peroxides, and the depletion of glutathione. Cadmium also has a very high affinity for glutathione and can form a complex with glutathione that is eliminated in bile. Cadmium also inhibits the activity of antioxidant enzymes, including catalase, manganese-superoxide dismutase, and copper/zinc-superoxide dismutase (Casalino et al., 2002). Cadmium-induced lipid peroxidation has been seen in animal studies in liver, kidney, brain, lung, heart and testis (Ercal et al., 2001). Cadmium can also substitute for zinc or selenium in metalloenzymes (Pope and Rail, 1995). Lowered levels of selenium as well as lowered activity of glutathione peroxidase (a selenium-dependent enzyme) have been seen in cadmium-exposed workers (Wasowicz et al., 2001). Cadmium's ability to generate free radicals also leads to expression of inflammatory chemokines and cytokines (Dong et al., 1998), the oxidation of nucleic acids, the alteration of DNA repair mechanisms, eventual cell death, and mutagenic changes involved in cadmium-induced cancers(Fowler,1978).

1.5 TOXIC EFFECTS OF CADMIUM

The acute oral lethal dose of cadmium for man has not been established; it has been estimated to be several hundred milligrams. Consumption by humans of fluids containing 13-15 mg of cadmium per liter has caused vomiting and gastrointestinal cramps (Fulkerson and Goeller, 1973).

Acute cadmium poisoning has occurred following exposure to fumes during the melting or pouring of cadmium metal. Fatalities have resulted from a 5-hour exposure to $8mg/m^3$ for 3 days although some individuals have recovered after exposure to $11mg/m^3$ for 2 hours. Acute pneumonitis resulted from inhalation of concentrations between 0.5 and 2.5 mg/m^3 for 3 days. Symptoms of acute poisoning include pulmonary oedema, headaches, nausea-vomiting, chills, weakness and diarrhea.

A syndrome called Itai-itai, first described in Japan, and has been associated with chronic ingestion of cadmium. The role that cadmium plays in the etiology of Itai-itai is not clear, and the dose of cadmium required to cause symptoms has not been determined. Symptoms of

the disease which occurred most often among elderly women, who had many children, are the same as those of osteomalacia (softening of the bone); the syndrome is characterized by lumbar pain, myalgia, and spontaneous fractures with skeletal deformation. It is accompanied by the classical renal effects of industrial cadmium poisoning: proteinuria and often glucosuria and aminoaciduria (Friberg et al., 1971). Daily cadmium intake by ingestion in the endemic area has been estimated to be 0.6mg (Schroeder et al., 1965), but it would have been considerably greater before 1955, when pollution control measures were instituted in the nearby mine. Epidemiological studies carried out in other parts of Japan indicate that the incidence of proteinuria is significant in cadmium-polluted areas.

1.5.1 Hypertension

It has been suggested that there is a relationship between cadmium and hypertension; (Perry et al., 1977; Balaraman et al., 1989).The results of several studies suggest that persons with hypertension have more cadmium and higher cadmium to zinc ratio in their kidneys than those without hypertension. In contrast to these findings, no relationship between hypertension and urinary cadmium excretion has been observed. (Szadkowski et al., 1969)
Oral administration of cadmium has produced hypertension in animals; the dose-response curve, however, is not monotonic. The greatest effects are observed with oral doses 0.01mg/day or intra-peritoneal injections of 0.0001 to 0.001mg/kg. Doses in order of magnitudes higher have little effect (Kopp et al., 1982).

The induction of hypertension in animals by feeding cadmium was first reported by Schroeder and Vinton (1962). Certainly parenteral administration of cadmium could induce acute transient hypertension in animals (Schroeder et al., 1966; Perry and Erlanger, 1971b). Schroeder did not believe that the growing incidence of arterial disease reflected the presence of such common and natural drinking-water constituents such as calcium bicarbonate, with which man has lived throughout history. What concerned the imaginative researcher was pollution by metals that modern man, the metallurgist, now scatters around him in profusion. What was needed was a laboratory free from metallic contamination. Schroeder constructed one high on a 1,600-ft. Vermont hill near his home base at Dartmouth Medical School. The building was all wood, with the nails sunk and sealed in. Anything that might contain lead or cadmium was excluded; the principal exception to the no-metal rule was stainless steel for

the cages that contained experimental rats and mice. Water pipes, where possible, were made of plastic. The pure mountain air was electrostatically filtered. Visitors were barred because they might carry metalliferous dust; even research-staff members had to take their shoes off before entering rooms. The animals were fed a diet with a meticulously defined metallic content, and their pure drinking water was superpurified. Among the 20 element that Schroeder investigated as potential artificial pollutants, cadmium produced the most striking result. Rats given minute traces of cadmium salts in drinking water all their lives developed high blood pressure of a type remarkably similar to the human disease. More females than males developed the disease, but it was deadlier to the males; the animals developed fatty plaques in their aortas, and showed enlargement of the heart. When rats receiving cadmium were divided into two groups, 80% of those on soft water developed high blood pressure as against only 17% of those on hard (calcium-containing) water. When the animals were treated with a drug that substituted zinc for the cadmium already in their tissues, blood pressures returned to normal. During the next years, Schroeder extended his initial observations in numerous reports (Schroeder, 1964; Schroeder and Buckman, 1967; Schroeder et al., 1968a; 1968b; 1970; Kanisawa and Schroeder, 1969a; 1969b). Perry and Erlanger (1971a) confirmed that chronically fed cadmium could raise systolic pressure. Schroeder et al., have reported very marked hypertension whereas Perry et al., could induce a mild elevation in blood pressure in experimental animals. Balaramam et al., (1989) conducted studies to find out the role of cadmium in producing hypertension and the mechanism for the same. He observed that in rats, acute i.v administration of $CdCl_2$, (0.5 and 1 mg/kg) produced a depressor response followed by a pressor response while the acute i.p administration of $CdCl_2$ (0.5 and 1 mg/kg) produced only a pressor response. Hexamethonium (10 mg/kg, i.v), phentolamine (5 mg/kg, i.v), propranolol (2 mg/kg, i.v) or indomethacin (20 mg/kg, i.p) did not modify the acute pressor response to $CdCl_2$, (i.v or i.p).Acute reserpinisation, bilateral adrenalectomy or chemical sympathectomy by guanethidine did not modify the acute pressor response to $CdCl_2$, (i.v or i.p). Verapamil(0.5, 1 and 2 mg/kg, i.v) or nifedipine (0.25 and 0.5 mg/kg, i.v) prevented the acute pressor response to $CdCl_2$, (i.v or i.p) it was hence concluded that cadmium might mimic calcium ion and produce a direct contractile effect on the vascular smooth muscle. Perry and Erlanger (1975) suggested direct vasoconstriction as a mechanism of cadmium-induced pressor effect.

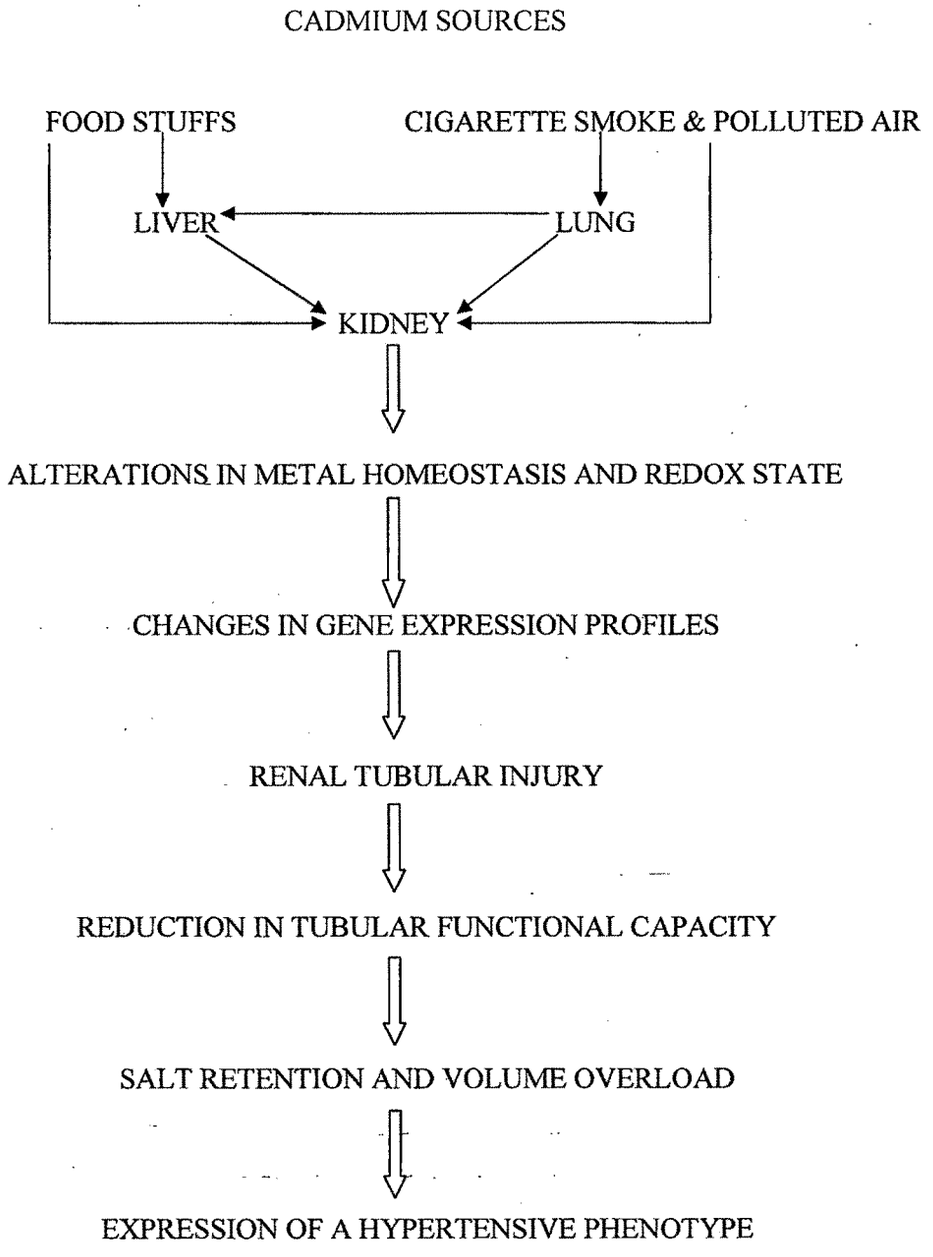
Fadloun and Leach (1981) suggested the involvement of sympathetic system and Caprino et al. (1982) showed the role of prostaglandins in the mechanism of acute pressor or the chronic hypertensive effect.

Although several mechanisms have been postulated, the precise mechanism(s) of Cd-induced cardiac impairment is still undefined. It has also been reported that cadmium may decrease or increase nitric oxide(NO) levels in endothelial cells and may enhance the production of reactive oxygen species leading to lipid peroxidation (LPO)(Chen et al.,2003;Cavicchi et al.,2000;Manca et al.,1991;Chevalier et al.,1994).Malondialdehyde (MDA) is the breakdown product of the major chain reactions leading to oxidation of polyunsaturated fatty acids and thus serves as a reliable marker of oxidative stress mediated LPO in myocardial tissue(Slater,1989;Sahna et al.,2003;Lefer and Granger,2000).

Schroeder et al. (1966) gave first report of chronic hypertension after the injection of cadmium. Cadmium acetate (2mg/kg, i.p.) was administered to female rats of the Long-Evans strain. Three weeks after a single injection of cadmium 31 percent of the rats exhibited elevation of blood pressure. When a second dose of cadmium 1mg/kg was given to the normotensives, all exhibited hypertension after one week. When cadmium was given intraperitoneally (2mg/kg) to another group of rats with partial constriction of left renal artery, 71 percent of the rats showed severe hypertension. Cadmium has been reported to induce hypertension accompanied by decreased vascular responsiveness to autonomic drugs in the rat and the rabbit. Cadmium acetate administration to female Sprague-Dawley rats failed to elevate blood pressure, although cadmium-treated animals exhibited decreased blood pressure responses to intravenously administered norepinephrine, acetylcholine, isoproterenol and atropine (Michael Porter et al., 1975). Fadloun and Leach (1981) have shown that female rats made hypertensive with cadmium chloride treatment exhibited increased sensitivity of the pressor response to noradrenaline. Nechay et al. (1978) have observed that rats treated chronically with low dose of cadmium showed potentiation of the pressor response to noradrenaline. Such discrepancies in vascular reactivity to various agonists in cadmium treated animals may be due to difference in the dosage, duration of treatment, route of administration and also species.

However, workers like Hammer et al. (1972) found no evidence of hypertension in workers exposed to cadmium containing superphosphate dust.

MECHANISM OF PRESSOR EFFECTS OF LOW DOSE CADMIUM



1.5.2 Respiratory toxicity

Chronic exposure to airborne cadmium results in a number of toxic effects; the two main symptoms are lung emphysema and proteinuria (Friberg, 1950). Emphysema appears after approximately 20 years of exposure; levels of exposure that result in disability have not been systemically determined. In one study, exposure to cadmium concentration of 3 to 15mg/m^3 produced emphysema.

1.5.3 Renal toxicity

An extensive review by Jarup et al (1998) includes an investigation of cadmium and renal damage. The highest load of cadmium is found in the renal cortex. Renal concentrations in second trimester fetuses and infants compared to autopsy studies in adults show renal cadmium concentration increases about 5,000 times from birth to adulthood. Studies of cortex concentrations have found that women have significantly higher concentrations than men, in spite of a higher male smoking rate.

The average cadmium exposure leads to kidney concentration of 20μg/g for nonsmokers and 40μg/g for smokers. At an average total intake of 30μg/day, it is estimated that renal tubular damage occurs in one percent of the population. At an intake of 70 μg (the World Health Organization provisional tolerable weekly intake), seven percent of adult population and up to 17 percent of high-risk groups would be expected to develop kidney lesions. A Belgian study examining kidney cadmium and renal damage estimates that 10 percent of the Belgian population may currently have kidney cadmium concentrations of 50 μg/g, resulting in early signs of renal damage, proteinuria, and calcium loss. In Japan, where cadmium exposure through environmental contamination of food and water has led to outbreaks of cadmium toxicity-related disease, cadmium induced tubular lesions have been identified in more that 20,000 people. In Swedish studies, early signs of renal damage have appeared in those with urine cadmium levels of 0.5-2.0 μg/g creatinine, corresponding to renal cortex concentrations of 10-40mg/kg, levels found in 50 percent of the adult Swedish population. Glomerular damage and kidney stones have been seen in those with occupational exposure to cadmium. Studies of workers with cadmium-induced renal damage estimate 40-80 percent increased annual mortality risk as a result of cadmium exposure and renal damage. A renal disturbance

that includes the excretion of low-molecular-weight proteins in the urine and an increase in amino acids and calcium. Study at autopsy has revealed that the principal renal effects of chronic cadmium poisoning are seen in the tubules but are pronounced only in the most severe cases (Kazantzis, 1979). It has been proposed that the minimum critical level of cadmium in the kidney required to produce renal tubular damage is approximately 0.2 mg/g. There is some evidence of an increase in the incidence of renal stones in those with prolonged exposure (Ahlmark, 1960). Infusion of cadmium chloride in dogs (plus cysteine to prevent effects on blood pressure etc) caused a drop in the sodium excretion and increased reabsorption of sodium in the proximal tubule. In addition to proteinuria, some cases have shown glycosuria. Once cadmium induced nephropathy is initiated it is irreversible.

1.5.4 Metabolic effects

Glucose tolerance curves in rats getting 17μg cadmium/ml of drinking water for 4 weeks (zinc and copper levels were varied) varied directly with serum and dietary zinc levels. Cadmium tended to lower serum zinc. Glucose tolerance was affected. Insulin levels also varied with zinc levels. Increasing intake of zinc prevented these adverse effects of cadmium (Fassette, 1975). Rats given cadmium chloride i.p. at 1mg/kg daily for 45 days showed a drop in hepatic glycogen and increased blood glucose and urea levels. Four enzymes involved in gluconeogenesis appeared to increase in the liver and renal cortex. The changes persisted for a month (Fassette, 1975).

1.5.5 Bones

In chronic cadmium exposure, bones may be secondarily affected or may be directly impaired before renal tubular damage develops (Kimura et al., 1974). The cadmium content of human bone in North America has increased by a factor of 50 in last 600 years. The majority of that increase is believed to have occurred in last 100 years. Classic cadmium poisoning (known as itai-itai disease in Japan) has been characterized by multiple fractures, osteomalacia, bone pain, and osteoporosis that occur along with renal disease. Animal studies indicate postmenopausal women may be at greater risk for cadmium-related bone loss and that cadmium may increase bone loss in women with pre-existing postmenopausal osteoporosis.

Epidemiological studies have found a positive correlation with elevated urinary cadmium levels and increased urinary calcium loss and elevated serum alkaline phosphatase levels. Studies have also found correlations between cadmium-induced renal tubular damage and bone loss. A study of 1,021 men and women, who had either worked at a factory or lived in a community in Sweden where nickel-cadmium batteries were produced, evaluated the relationship of cadmium exposure to kidney and bone disease. Those who were environmentally exposed and had the highest blood cadmium levels had a four-fold risk of tubular proteinemia. Older individuals (over 60 years) in that group had a three-fold risk of significant bone loss (Z-score <-1) compared to the same-age group with no known cadmium exposure. The Z score results from a comparison to the average bone density scores of a group of similar-aged individuals. A score of less than 0 indicates bone loss greater than the average of that same group.

The mechanisms behind cadmium and bone loss are related to renal tubular cell damage that results in elevated levels of urinary calcium and lowered levels of 1,25 dihydroxy-cholecalciferol, a consistent finding in women environmentally exposed to significant levels of cadmium. Lower levels of vitamin D_3 alter calcium homeostasis by decreasing absorption of calcium in the gut and altering deposition in bone.

1.5.6 Liver

Liver function can be lowered by severe exposure; however, there have been few reports on liver diseases resulting from occupational exposure to cadmium (National Institute for Occupational Safety and Health, 1977)

1.5.7 Teratogenic and Reproductive effects

Oral doses of 10mg/kg per day for six weeks have produced birth defects in rats (Sutou et al., 1980). Cadmium appears to be completely filtered by the placenta when adequate zinc and copper are available for the induction of metallothionein. Studies with newborn rats reveal newborns whose cadmium-exposed mothers had been given adequate zinc and copper diet during pregnancy were cadmium free at birth, as opposed to newborns whose cadmium-exposed mothers had a zinc and copper-deficient diet (Goyer and Cherian, 1992). Maternal hypertension and low birth weight have been associated with elevated cadmium levels in

infants (WHO, 1992). Environmental exposure to cadmium in pregnant women has been correlated with increased levels of lipid peroxides, and the incidence of threatened spontaneous abortion, toxemia, and anemia (Tabacova et al., 1994).

1.5.8 Carcinogenicity

Cadmium is classified as a group I human carcinogen, meaning sufficient evidence for carcinogenesis has been found in both animals and humans. Occupational and environmental exposure has been shown to increase risk for lung cancer with co-exposure to arsenic, and renal cancer with cadmium exposure alone. While animal studies support a role for cadmium-induced prostate cancer, inconsistent findings exist for cadmium's role in human prostate, breast, testicular, and bladder cancers. In animal studies, cadmium compounds have produced sarcoma at the site of injection and also interstitial cell tumors in the testes of rats (Oldiges and Glaser, 1986). Inhalation of cadmium chloride aerosol has produced high incidence of primary lung cancer in rats (Oldiges and Glaser, 1986). However the administration of cadmium salts by ingestion has not given rise to cancer (Kazantzis, 1963).

Epidemiological studies of workers occupationally exposed to cadmium have provided only inconclusive evidence that such exposure increases the risk of lung, prostate and renal cancers. The results are difficult to interpret because of such confounding factors as the incidence of smoking and exposure to other possible carcinogens (Kazantzis, 1963). The U.S. Environmental Protection Agency has concluded that cadmium inhalation is dose-related to lung cancer in exposed workers but there is no evidence that cadmium is carcinogenic by ingestion (U.S. Environmental Protection Agency, 1985).

1.5.9 Immunity

Cadmium has shown immunomodulatory activities (Krocova et al., 2000). The viability of both lymphocytes and macrophages was affected by heavy metals in a dose-and time-dependent manner. Cadmium suppressed the production of N-oxides but stimulated significantly the proliferation of spleen cells. Cadmium seemed to trigger the Th2 cytokine regulatory pathway [interleukin 4 (IL-4), interleukin 10 (IL-10)]. The results suggest the metal-induced changes in immunoregulatory mechanism of host with potentially severe clinical consequences.

1.5.10 Neurotoxicity

It has been found that neonatal animals are more susceptible to the neurotoxic effects of cadmium compared with the adults. Rohrer et al. (1978) reported changes in the cerebral vasculature of the foetus exposed to cadmium. Cadmium accumulates in the adult brain although it penetrates the blood brain barrier with more ease in fetal rats. Greater concentration of cadmium was found in brains of newborn rats after intravenous administration indicating that the blood brain barrier to cadmium is not fully developed in the neonates (Wong and Klaassen, 1981). Rozear et al. (1971) reported decreased spontaneous neural firing after administration of the heavy metal into the cerebral cortex or brain stem of cat. Further, *in vitro* addition of this metal was found to act as a synaptic blocking agent at both adrenergic and cholinergic synapses (Coopper et al., 1978).

Ribas-Ozzonas et al. (1974) reported an increase in 5-hydroxytryptamine and 5-hydroxyindole acetic acid in different regions of rat brain following 60 min of a single intraventricular injection of cadmium. Shukla and Singhal (1984) have shown that the administration of 0.5mg cadmium/kg/day i.p. to 22 day old rats for 30 days was found to increase NA in the midbrain and pons-medulla, and 5-HT in the hypothalamus region without affecting dopamine levels. However, a dose of 0.1mg cadmium/kg did not produce any change in the levels of monoaminase but decreased GABA contents in the cerebellum and hypothalamus regions. In contrast the administration of 0.5mg cadmium/kg/day i.p. to adult rats has been reported to increase dopamine and decrease 5-HT levels in the whole brain (Shukla and Chandra, 1981). Further, *in vitro* addition of cadmium inhibited synaptosomal uptake of ^{3}H dopamine and ^{3}H norepinephrine in a concentration dependant manner (Hobson et al., 1983). Exposure to cadmium has also been found to alter the behavioral responses to apomorphine in rats suggesting the existence of dopamine supersensitivity in metal exposed animals (Smith et al., 1983a). It was proposed that the cadmium-induced increase in spontaneous locomotor activity might be related to increased adrenergic activity. However, the precise mechanism by which cadmium alters behavior and produces neurotoxic manifestations remains to be elucidated.

Table 1.2: Multiplicity of cadmium targets and toxicities in human subjects

Cadmium targets	Toxicities	References
Kidney	Tubular and glomerular dysfunction, chronic renal failure, stone, hypertension, depressed renal synthesis of active vitamin D, cancer	Buchet et al., (1990); Yamanaka et al., (1998);Cai et al., (2001); Satarug et al., (2003, 2006); Baker et al., (2005);
Bone	Osteoporosis	Staessen et al., (1999); Alfven et al.,(2000, 2002); Honda et al., (2003a); Aoshima et al.,(2003); Jin et al., (2002)
Peripheral vascular tissues	Atherosclerosis	Navas-Acien et al., (2005)
Liver	Increased risk of deranged drug metabolism and liver cancer associated with environmental exposure to procarcinogens attributable to cadmium induction of CYP2A6, CYP2E1 and CYP2C9 in liver	Baker et al., (2001, 2003, 2005); Satarug et al., (2006)
Mammary gland	Reduction in calcium secretion in milk	Nishijo et al., (2002), Honda et al., (2003b)
Placenta	Impaired placental metal transport function, increased risk of preterm delivery	Nishijo et al., (2002)
Prostrate, breast, colon, pancreas	Malignant disease	Ekman, (1999)

1.6 FREE RADICALS AND OXIDATIVE STRESS

1.6.1 Free Radicals

Free radicals are energetically unstable and highly dangerous molecules which are constantly generated during body functions such as respiration, oxidative energy metabolism and immune activity. Free radicals are also produced from other sources (UV radiation, smoke, pollution, heavy metals, rancid fatty acids, etc). The destructive effects of free radicals are far reaching, including: cell membrane destruction via the interaction of fatty acids with oxygen to form dangerous peroxides (lipid peroxidation) genetic damage via DNA mutation; decline in immune function; increase in inflammatory conditions; growth and spread of cancer;

oxidation of LDL cholesterol, leading to atherosclerosis; hormone disruption, contributing to diabetes and other systemic disorders.

A free radical can be defined as a chemical species possessing an unpaired electron. It can also be considered as a fragment of a molecule. Free radicals can be formed in three different ways:

a) By hemolytic cleavage of a covalent bond of a normal molecule, with each fragment retaining one of the paired electrons;
b) By the loss of a single electron from a normal molecule;
c) By the addition of a single electron to a normal molecule.

It can be a source of confusion that the electron in one of the most important molecule in free radical biochemistry, oxygen, is distributed in such a way that two of the electrons are unpaired. Thus, oxygen is sometimes considered a di-radical. While the di-radical nature of the oxygen does enable it to react with many other free radicals, in general it reacts relatively slowly with non-radical species. Normally a balance between oxidative events and antioxidative forces maintains the status quo within living cells. When the normal balance is upset, either by loss of reducing agent or protective enzymes or by increased production of oxidizing species, or by both events simultaneously, the tissue is considered to be in oxidant stress. Oxidative stress is caused by exposure to these reactive oxygen intermediates, such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) that can damage proteins, nucleic acids and cell membranes Increasing evidence suggests that the cumulative damage caused by reactive oxygen species contributes to numerous diseases(Aruoma and Halliwell, 1998). Oxidative stress is an unavoidable by product of the aerobic lifestyle because of superoxide and hydrogen peroxide radicals, which are formed wherever molecular oxygen chemically oxidizes electron carriers. This superoxide concentration is tolerable, about half what is necessary to diminish the activities of vulnerable enzyme and inhibit cell growth. Thus the defenses maintained in the cell are calibrated to just avoid toxicity from endogenous oxidants. These defenses are inadequate, however, if the rates of intracellular $O_2^{\cdot-}$ and H_2O_2 formations are accelerated. $O_2^{\cdot-}$ and H_2O_2 have different chemical reactivity and generate distinct type of damage inside cells.

1.6.2 Oxygen Free Radicals or Reactive Oxygen Species (ROS)

1.6.2.1 Superoxide radical:

Arguably the most important of free radicals in biological systems are radical derivatives of oxygen. Reduction of oxygen by transfer of a single electron to it will produce the superoxide free radical anion (Saran et al., 1989)

$$O_2 + e^- \rightarrow O_2^{\cdot -}$$

Superoxide is formed in almost all aerobic cells, a major source being the respiratory burst of phagocytic cells, when they contact foreign particles or immune complexes. Some of the superoxide production in the cells is accidental e.g. the amount of 'leakage' onto oxygen of electrons from various components of the cellular electron transport chains, such as those of mitochondria, chloroplast and endoplasmic reticulum. The rate of superoxide production rises, as the oxygen concentration increases. Superoxide anion radical is metabolized by the metalloenzymes superoxide dismutase (SOD) to form hydrogen peroxide and molecular oxygen (Cheeseman and Slater, 1993).

1.6.2.2 Hydrogen Peroxide:

A two-electron reduction of oxygen would yield hydrogen peroxide.

$$2 + 2e^- + 2H^+ \rightarrow H_2O_2$$

Hydrogen peroxide is often generated in biological system via the production of superoxide: two superoxide molecules can react together to form hydrogen peroxide and oxygen.

$$O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide is not a free radical but falls into the category of 'reactive oxygen species' (ROS) that include not only oxygen free radical but also non-radical, oxygen derivatives that are involved in oxygen radical production (Cheeseman and Slater, 1993). Hydrogen peroxide is lipid soluble and thus very diffusible with and between the cells.

1.6.2.3 Hydroxyl radical:

Three-electron reduction state, formed by Fenton reaction and decomposition of peroxynitrite. It is an extremely reactive oxidizing radical that will react with most biomolecules at diffusion controlled rates. It thereby will not diffuse a significant distance within a cell before reacting. Hydroxyl has an extremely short half-life but is capable of causing great damage within a small radius of its site of production. It combines with almost all molecules found in living cells. It is so reactive that no enzyme system involving it as a substrate exists. Because of its reactivity, the hydroxyl radical does not travel far and has a half-life of a few microseconds. However, hydrogen peroxide can cross cell membranes and lead to hydroxyl radical formation at more distant sites.

1.6.2.4 Singlet Oxygen:

Besides superoxide and hydroxyl radicals, there are some other ways by which increased reactivity of oxygen have been noticed. This is by lifting one of the unpaired electrons to an orbital of higher energy with an inversion of spin. There are two forms of singlet oxygen, delta and sigma, corresponding to the first and second excited states respectively.

Singlet oxygen is produced under various pathophysiological conditions in mammalian tissues. Excitation of oxygen to singlet state can be achieved when several pigments are illuminated in the presence of oxygen. Singlet oxygen formation is thus likely to be generated in tissues by photochemical reactions (photoexcitation) involving endogenous pigments and xenobiotics. Besides photoexcitation reactions, singlet oxygen is also generated in the tissues by dark reactions (chemo excitation) consisting of enzymatic reactions and radical interaction.

1.6.2.5 Nitric oxide:

This free radical has a structure similar to that of superoxide, except that it has two electrons less. The endothelium derived relaxing factor (EDRF) produced by vascular endothelium, which is an important mediator of vascular responses induced by several pharmacological agents is nitric oxide. The free radical nitric oxide is synthesized by vascular endothelial cells, phagocytes, certain cells in the brain and many other cell types from the amino acid, L-

arginine, in the presence of nitric oxide synthase. Nitric oxide acts as a neurotransmitter, prevents platelet aggregation, and is a defense molecule of the immune system against tumor cells, parasites and bacteria. Nitric oxide can react with superoxide anion to generate peroxynitrite, which is a damaging species that can get protonated and undergo decomposition to hydroxyl radical, causing additional damage (McCord, 1991).

1.6.2.6 Hypochlorous acid:

Hypochlorous acid (HOCl) is not a free radical but a potent chlorinating and oxidizing agent. It mainly attacks primary anions and sulphydryl groups in proteins and may chlorinate purine bases in DNA. One of its important targets is α-proteinase, which is the major inhibitor in body fluids.

1.6.3 Evidence Of Role Of Free Radicals

1- A large body of comparative biochemical evidence suggests that longer lived species exhibit decreased levels of oxidative damage, decreased susceptibility to oxidative stress, and decreased generation of reactive oxygen species.

2- Results have demonstrated that the over expression of catalase, an enzyme involved in the decomposition of hydrogen peroxide, increased both the average lifespan and maximum lifespan of mice by 20%.

3- Mutant strains of the round worm Caenorhabditis elegans that are more susceptible to free radicals have shortened life span, and vice versa. However, increasing atmospheric oxygen tension above the normal 21% O_2 does not meaningfully decrease life span of C.elegans.

4- Drosophila that have mutations in enzymes relating to reactive oxygen species metabolism have also been shown to have dramatically reduced life-spans, increased susceptibility to oxidative stress and ionizing radiation, partial female and complete male sterility, and a general "enfeebled" phenotype characterized by deformed wings and abdomen.

5- While genetic manipulations that increase the levels of oxidative damage generally do shorten life span in mice, there is at present very limited evidence that decreasing free radicals below their normal levels actually extends average or maximum life span.

6- Consumption of high levels of antioxidants, which should increase life span under the theory, may extend average but not maximum life span in mice. The effect, if present, is weak and only inconsistently observed.

7- Phenybutylnitrone (PBN) was shown to produce about a 10% extension of maximum life span in experimental animals (Saito et al., 1998), in one laboratory; however, this finding has not been reproduced by other laboratories.

8- Antioxidant supplementation has not been conclusively shown to produce an extension of life span in a mammal.

9- With the increasing acceptance of free radicals, they have been implicated in a very large number of human diseases. In recent years, reports confirming the damaging role of the reactive oxygen species in various diseases to human beings and animals like cancer (Guyton and Kensler, 1993), ischemia (Flitter,1993), diabetes (Wolff, 1993), liver damage (Poli, 1993), atherosclerosis (Esterbauer et al., 1993), modification of LDL (Steinbrecher, 1987) have been published.

Fig 1- SCHEMATIC DIAGRAM OF CELLULAR MECHANISM OF FREE RADICAL GENERATION AND CELL DAMAGE.

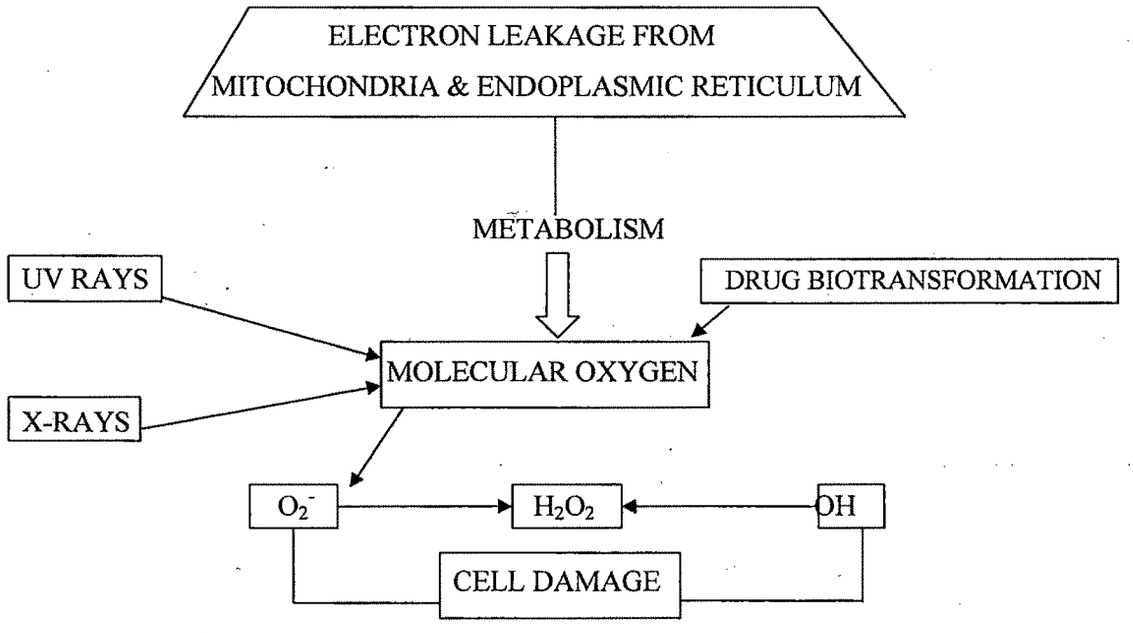


Fig 2- STEPS OF FREE RADICAL INDUCED CELL DAMAGE

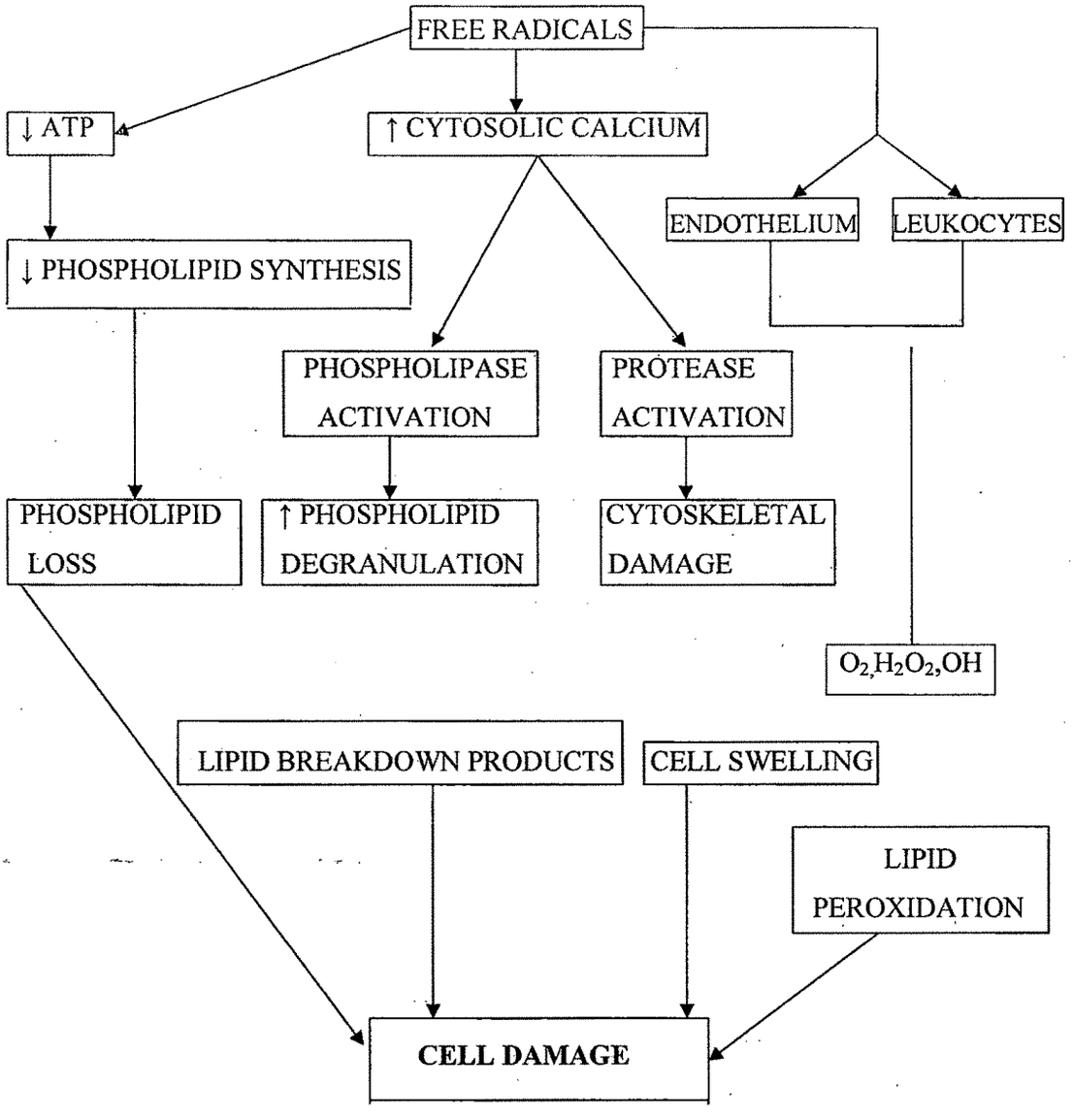


FIG 3- MECHANISM BY WHICH FREE RADICALS CAUSE DNA DAMAGE:

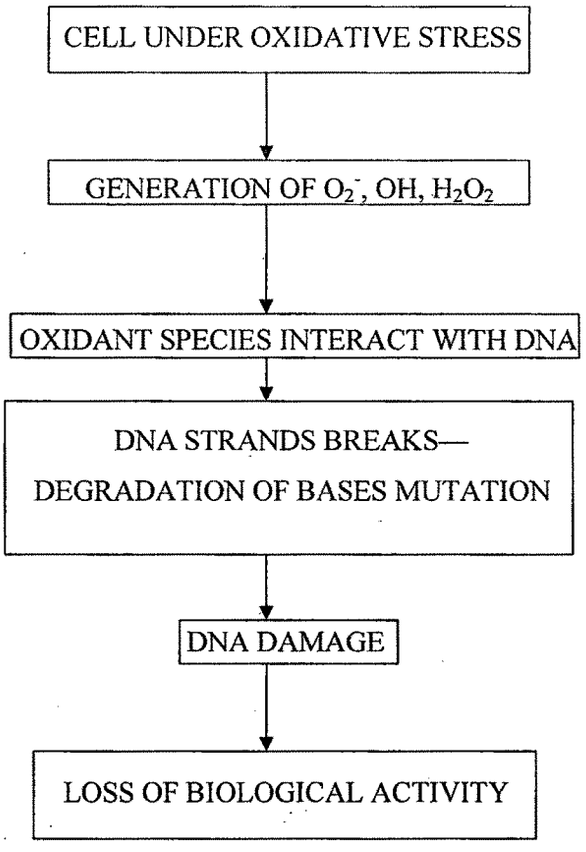


FIG 4- MECHANISM BY WHICH FREE RADICALS DAMAGE PROTEINS

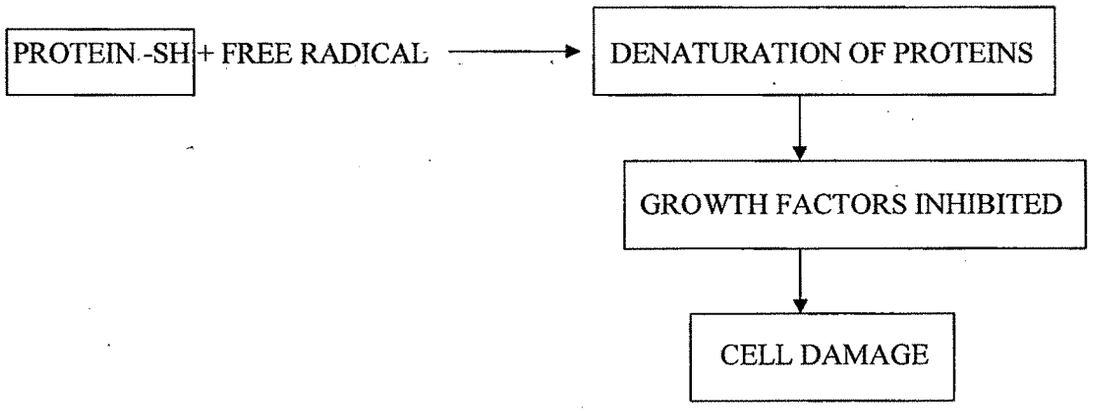


FIG 5- ROLE OF FREE RADICALS IN CARCINOGENESIS

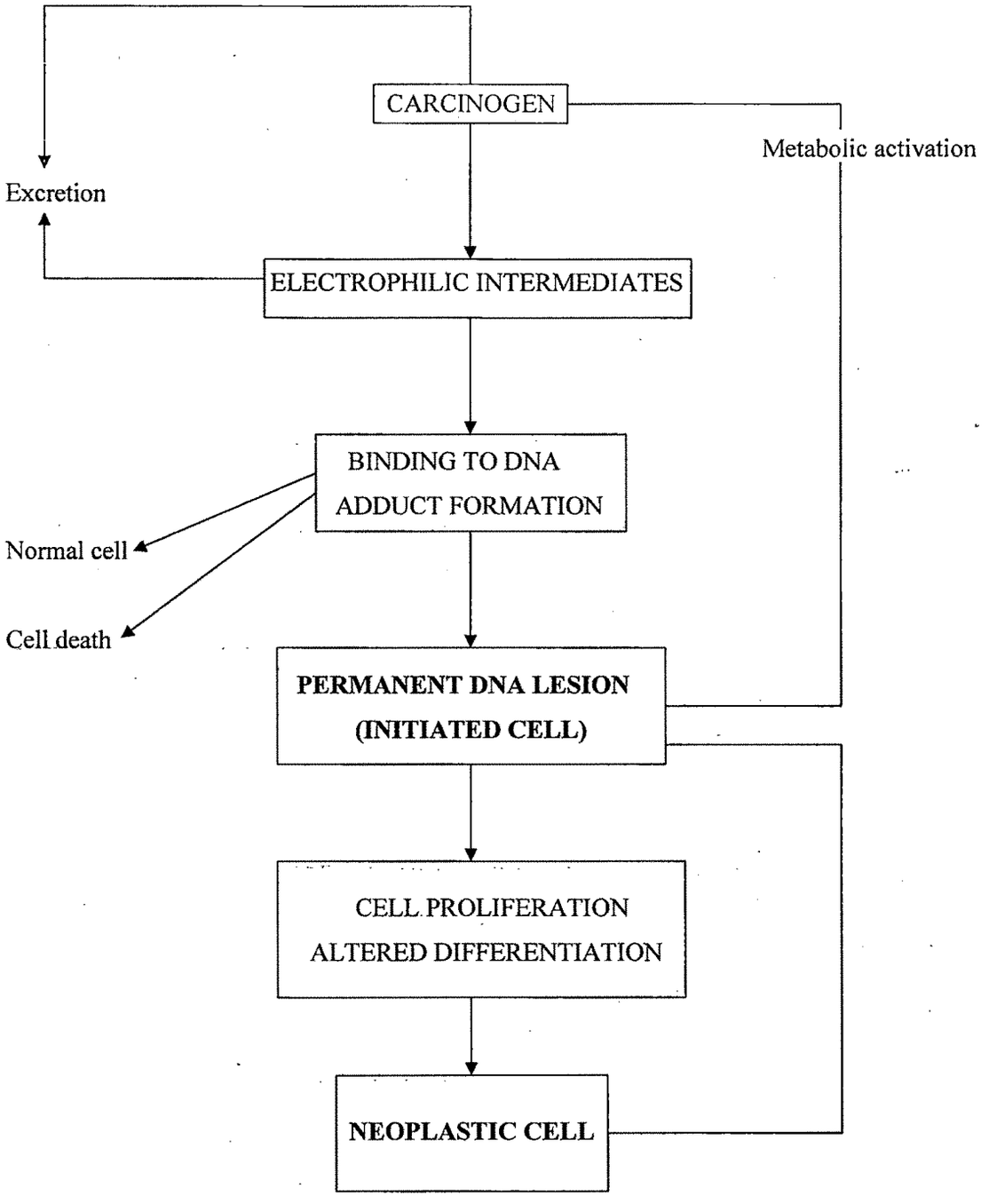
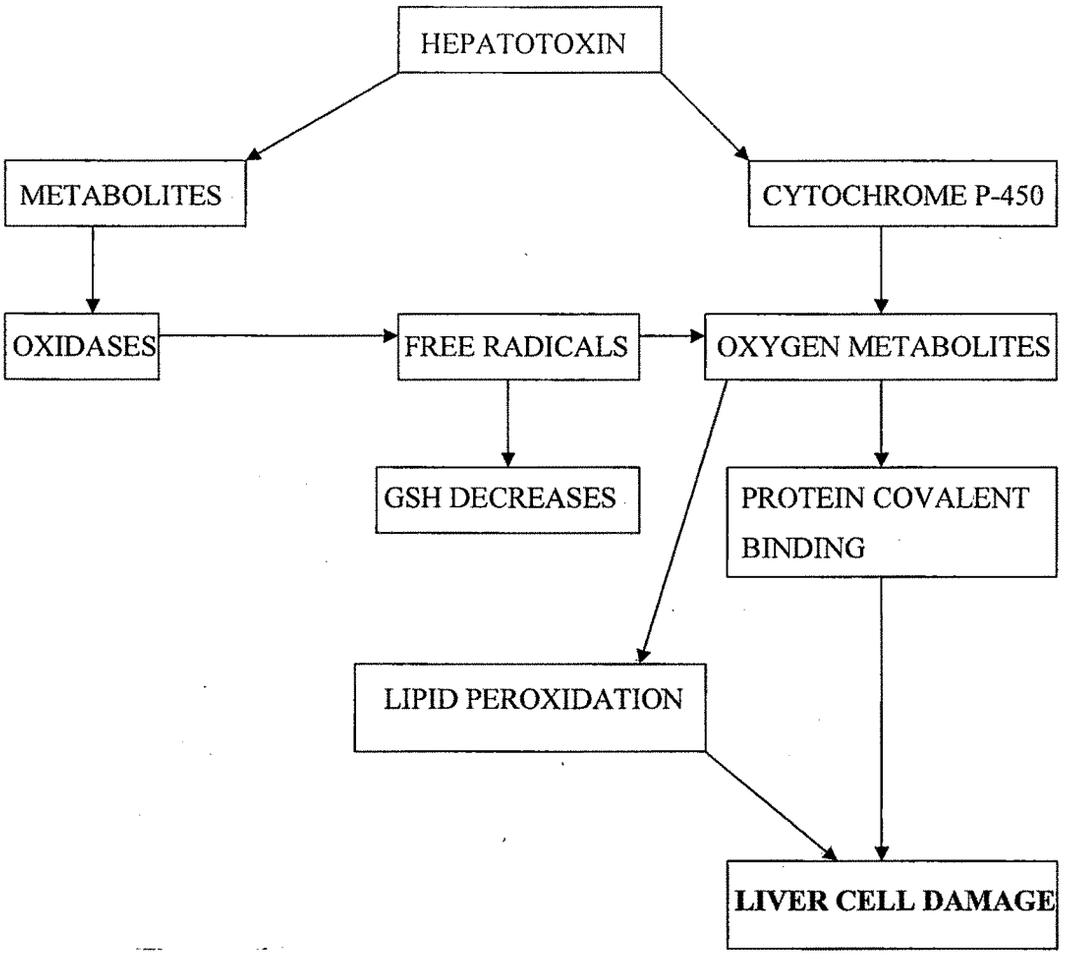
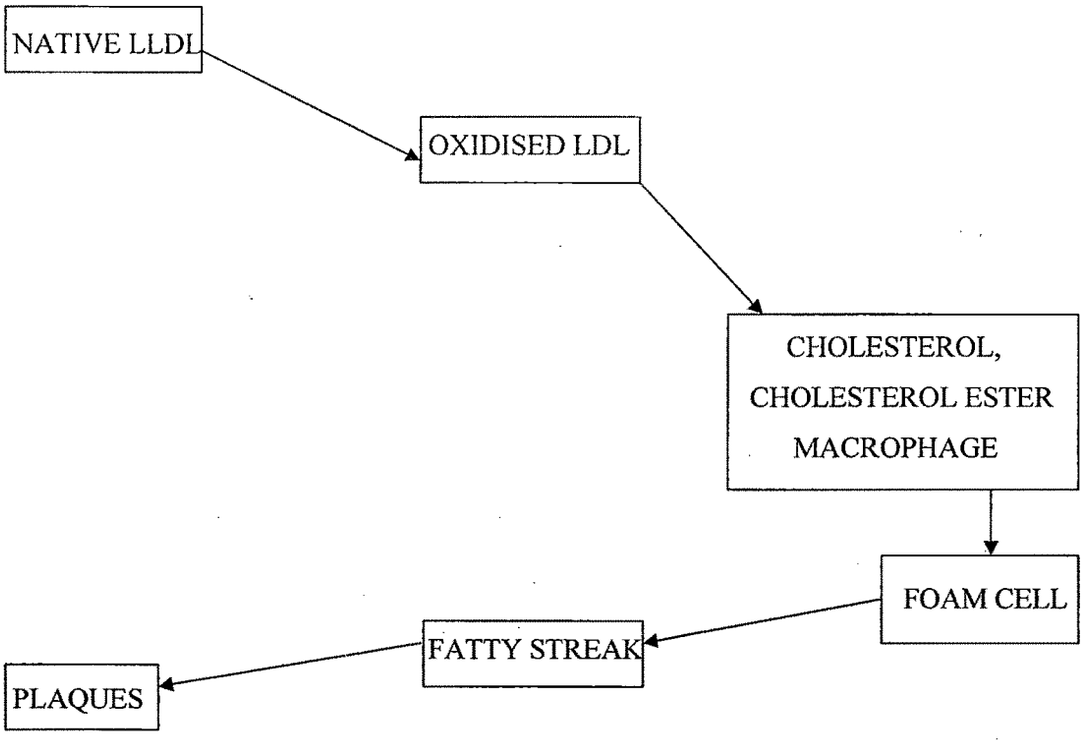


FIG 6- ROLE OF FREE RADICALS IN LIVER DAMAGE



Hepatotoxins cause hepatic cell injury either by direct covalent binding to the membrane protein and lipids, or more commonly by the formation of reactive free radicals. Hepatotoxins like acetaminophen acts by both oxidative damage and covalent binding. The drug is detoxified in the liver through sulfation and glucoronidation and is converted to toxic metabolite by cytochrome P-450. When large doses are ingested, the metabolites accumulate in the cell owing to GSH depletion and covalently bind proteins and nucleic acids, thus increasing drug toxicity and resulting in massive liver necrosis.

FIG 7- ROLE OF FREE RADICALS IN ATHEROSCLEROSIS



Lipid Peroxidation involved in the oxidative modification of low density lipoprotein (LDL) ultimately results in the formation of atherosclerotic lesions. In early atherosclerotic lesions lipid-laden 'foam cells' are found to have accumulated in the subendothelial space. As the disease progresses most of the cells die, generating fatty streaks that may ultimately develop into plaques.

Usually LDL is taken up by the LDL receptors. Oxidized LDL is not recognized by LDL receptors. These oxidized LDL are taken up monocyte-macrophage system (a scavenger system) by phagocytosis. Increased oxidized LDL within macrophage system releases lipid peroxide products that irritates the endothelium and causes platelet aggregation forming a plaque.

1.6.4 `Mechanism of Oxidative Stress

Oxidative stress is produced in cells by oxygen-derived species resulting from cellular metabolism and from interaction with cells of exogenous sources such as carcinogenic compounds, redox-cycling drugs and ionizing radiations.

1.6.4.1 DNA damage and Gene activation:

DNA damage caused by oxygen derived species is the most frequent type encountered by aerobic cells. When this type of damage occurs to DNA, it is called oxidative DNA damage and it can produce a multiplicity of modification in DNA including base and sugar sections, strand breaks, DNA-protein cross links and base-free sites. Accurate measurement of these modifications is essential for understanding of mechanisms of oxidative DNA damage and its biological effects. Numerous DNA lesions have been identified in cells and tissues at steady state levels and upon exposure to free radical-generating systems. Data accumulated over many years clearly show that oxidative DNA damage plays an important role in a number of disease processes. Thus, oxidative DNA damage is implicated in carcinogenesis and neurodegenerative diseases such as Alzheimer's disease. There is also strong evidence for the role of this type of DNA damage in the aging process. The accumulation of oxidative DNA damage in non dividing cells is thought to contribute to age associated diseases. DNA damage is countered in cells by DNA repair, which is a basic and universal process to protect the genetic integrity of organisms. The genomes of organisms encode DNA repair enzymes that continuously monitor chromosomes to correct DNA damage. Multiple processes such as base and nucleotide-excision pathways exist to repair the wide range of DNA damages. If left unrepaired, oxidative DNA damage can lead to detrimental biological consequences in organisms, including cell death, mutations and transformation of cells to malignant cells. Therefore, DNA repair is regarded as one of the essential events in all life forms. There is increasing evidence of the importance of oxidative DNA damage and its repair to human health. Thus, it becomes exceedingly important to understand, at the fundamental level, the mechanisms of oxidative DNA damage, and its processing by DNA repair enzymes as well as how unrepaired DNA lesions may lead to cytotoxicity, mutagenesis and eventually to diseases and aging. More detailed knowledge of mechanisms of DNA damage and repair might allow us to modulate DNA repair. This could lead to drug developments and clinical

application including the improvement of cancer therapy by inhibiting DNA repair in drug or radiation resistant tumors and/or the increase in the resistance of normal cells to DNA damage by over-expressing DNA repair genes.

The ability of free radicals to activate the transcription is now recognized. The immediate early genes c-fos, c-myc, c-jun and beta actin are induced rapidly by oxygen free radicals, possibly through the induction of DNA strand breaks. These genes encode transcription factors, which participate in the induction of cell growth, differentiation and development.

1.6.4.2 Lipid damage:

Lipids are the most susceptible targets of oxidative stress caused by free radicals. Cell membranes which are a rich source of polyunsaturated fatty acids (PUFAs) are readily attacked by oxidizing radicals and their destruction is known as lipid peroxidation. Lipid peroxidation is particularly damaging because it proceeds as a self-perpetuating reaction (Cheeseman and Slater, 1993) consequent to free radical production in cell because it is a destructive chain-reaction that can directly damage the structure of the membranes and indirectly damage other cell components by the production of reactive aldehydes.

1.6.4.3 Damage to Enzyme and Proteins:

Metal catalyzed oxidation has been identified as a post translational covalent modification of proteins which may be important in several physiological and pathological processes. Proteins are also membrane constituents and hence their damage explains the membrane damaging effects of free radicals. As yet, convincing evidence of the involvement of free radical induced damage to proteins as an important mechanism in a tissue injury or disease process is lacking though oxidative damage to eye lens has long been suggested to be involved in cataract (Stadtman, 1990; Spector, 1985; Seccia et al., 1991; Simpson et al., 1992).

1.6.4.4 Damage to Carbohydrates:

Sugars including glucose, mannitol and deoxysugars react readily with hydroxyl radical. Hyaluronic acid, which forms the central axis of protoglycans and maintains the viscosity of

synovial fluid, is fragmented following exposure to free radicals resulting in destabilization of connective tissue and loss of synovial fluid viscosity.

1.6.4.5 Lipid Peroxidation (LPO)

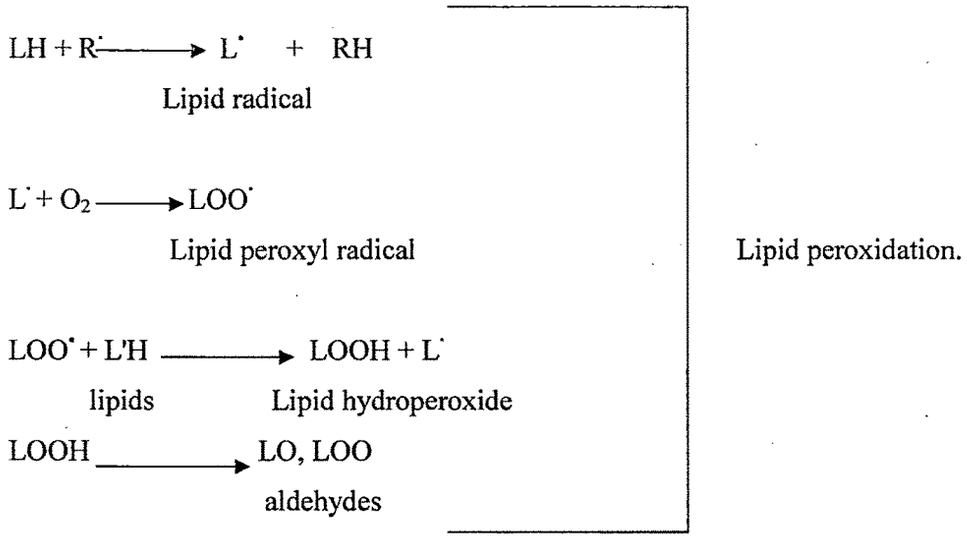
Lipid peroxidation (LPO) is defined as the 'Oxidative deterioration of polyunsaturated fatty acid (Tappel, 1973). It involves the formation and propagation of lipid radicals, the uptake of oxygen, a re-arrangement of the double bond in unsaturated lipids that results in the variety of degraded products like alkenes, malondialdehyde, lipid hydroperoxide and conjugated diene and eventually destruction of membrane lipids. Biological membranes are often rich in the unsaturated fatty acids and bathed in an oxygen rich, metal containing fluid. Therefore the membrane lipids are more susceptible to peroxidative attack.

The two major systems of lipid peroxidation in the liver are enzymatic and non-enzymatic lipid peroxidation. The enzymatic lipid peroxidation is mediated by the NADPH, cytochrome-c-reductase (Pederson and Aust, 1972) and non-enzymatic LPO is mediated by the transition metal ions like iron and copper (Ottolenghi, 1959). LPO has a profound effect on the macromolecules, RNA, DNA and other substances, which possess amino groups and reacts with malondialdehyde (Tappel, 1973). Nucleic acids are also damaged by direct reactions with lipid radical (Nielsen, 1981). The mechanism is thought to occur via single and double strand breaks in DNA, which leads to mutagenesis, carcinogenesis and cell death (Seholes, 1983; Kensler and Trush, 1984). Ionizing radiation produces 60-70 percent breakdown of DNA strand, chromosomal aberrations, mutations and cell killing (Okada et al., 1983)

Lipid Damage

Lipids are by far the most susceptible targets for free radical attack. Cell membranes are rich source of polyunsaturated fatty acids (PUFAs), which are readily attacked by oxidizing radicals. The oxidative destruction of PUFAs, known as lipid peroxidation, is particularly damaging because it proceeds as a self perpetuating chain reaction (Cheeseman and Slater, 1993). The general process of lipid peroxidation can be envisaged as in the scheme below, where LH is the target PUFA and R$^{\cdot}$ the initiating, oxidizing radical. Oxidation of the PUFA generates a fatty acid radical (L$^{\cdot}$) that rapidly adds oxygen to form a fatty acid peroxy radical

(LOO˙). The peroxy radicals are the carriers of the chain reaction, they can oxidize further PUFA molecules and initiate new chains, producing lipid hydroperoxides (LOOH) that can break down to yet more radical species and to wide range of compounds, notably aldehydes (Porter, 1990; Esterbauer et al.,1991b).



The breakdown of lipid hydroperoxides often involves transition metal ion catalysis, yielding lipid peroxyl and lipid alkoxyl radicals. Aldehydes are always formed when lipid hydroperoxides break down and many of them are biologically active, particularly a class known as the hydroxynoneal (Esterbauer et al., 1988; 1991b).These compounds can diffuse from the original site of attack and spread the damage to other parts of the cell.

In summary, lipid peroxidation is of particular significance as a damaging reaction consequent to free radical production in cell because; first, it is very likely to occur due to the availability and susceptibility of PUFA in membranes. Secondly, it is destructive chain-reaction that can directly damage the structure of the membrane and indirectly damage other cell components by the production of reactive aldehydes.

The detection and measurement of lipid peroxidation is most frequently cited to support the involvement of free radical reaction in toxicology and human diseases. Lipid peroxidation

has been implicated in a wide range of tissue injuries and diseases (Cheeseman and Slater, 1993).

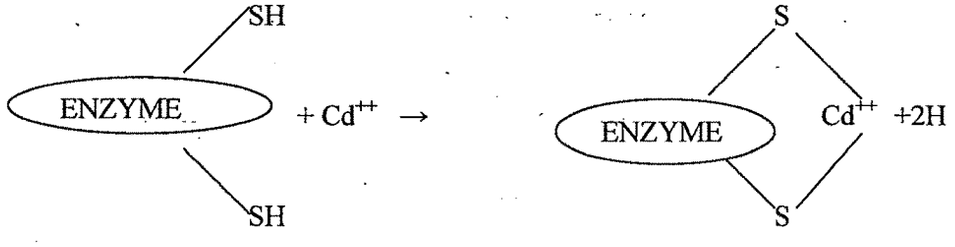
1.6.5 Measurement of Oxidative Stress

There is increasing evidence to support the involvement of free radicals in several diseases and disorders. The increasing interest in the role of free radicals in the pathogenesis of different diseases has lead to increased need to measure the free radicals and their reactions. The major problems in determination of most of the free radicals of interest are that they are extremely reactive and possess very short life. It is completely impossible for a reactive free radical produced in an internal tissue and having a lifetime measured in microseconds to diffuse into the blood such that it can be detected at a distant time.

Thus, free radical activity is usually assessed by indirect methods such as measurement of the various end products resulting from interaction of free radicals with cellular components such as lipids, protein and DNA (Pryor and Godber, 1991; Slater, 1984)

1.6.6 Metals And Oxidative Stress

Metal ions play a major role in propagating free radical reactions and consequently in determining the degree of free radical pathology. Metal complexes especially in a non-polar environment are particularly effective in the initiation of lipid peroxidation.Transition metal ions such as Fe^{3+}, CO^{3+}, Ce^{4+}, Mn^{3+}, Cu^{2+} and Cd^{2+} are able to oxidize various substrates by one electron withdrawal to form cation free radicals. In general, toxic metals attack the active sites of enzymes and inhibit essential enzyme function. Heavy metal ions, in particular Pb^{2+}, Cd^{2+}, Hg^{2+} and Ar^{2+} acts as an effective enzyme inhibitor.



Metalloenzyme contains metal in their structures. Their action is inhibited when one metal ion of a metalloenzyme is replaced by another metal ion of similar size and charges. Thus, zinc in some metalloenzymes is substituted by cadmium, which leads to toxicity. The enzyme inhibited by cadmium includes adenosine triphosphate, alcohol dehydrogenase, carbonic anhydrase etc. Recent studies (Stohs and Bagchi, 1995) have reported that the metals, including iron, copper, chromium and vanadium, undergo redox cycling; while cadmium, mercury, nickel as well as lead depletes glutathione, protein-bound sulfhydryl groups resulting in the production of reactive oxygen species such as superoxide ion, hydrogen peroxide and hydroxyl radicals. As a consequence, enhanced lipid peroxidation, DNA damage and altered calcium and/or sulfhydryl homeostasis occurs. Phagocytic cell may be another important source of reactive oxygen species in response to metal ions. Thus the ability to generate reactive oxygen species by redox cycling of quinones and related compounds may require metal ions. Metal ions may also enhance the production of tumor necrosis factor alpha (TNF alpha) and activate protein kinase C as well as induce the production of stress proteins (Kohler and Eckwert, 1997).

1.7 ANTIOXIDANTS:

1.7.1 Introduction

An antioxidant is any substance that when present at low concentration compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate. The term oxidizable substrate includes almost everything found in living cells, including proteins, lipids, carbohydrates and DNA. The term reactive oxygen species is a collective one that includes not only oxygen centered radicals such as superoxide radical, singlet oxygen and hydroxyl radical, but also some potentially dangerous non-radicals like hydrogen peroxide. Fortunately, the human body makes several important antioxidants. The most important are ubiquinone and glutathione. Enzymes such as superoxide dismutase, catalase and glutathione peroxidase also destroy free radicals. Antioxidants may act at different levels in the oxidative process by scavenging initiation of free radicals, binding metal ions, scavenging peroxyl radicals and removing oxidatively damaged bio-chemicals. Some antioxidants must be provided as micronutrients; they include ascorbic acid, beta-carotene, Coenzyme Q10, Vitamin E, Alpha Lipoic acid and trace metals such as selenium.

1.7.2 Mechanism of Antioxidants

The two main mechanisms by which Antioxidants act:

A-SEQUESTRATION OF TRANSITION METALS:

Iron and copper ions are in chemical forms that can decompose hydrogen peroxide (H_2O_2) to hydroxyl radicals. The human body is very careful to ensure that as much iron and copper as possible is kept safely bound to transport or storage proteins (transferrin, hemosiderine, ferritin, ceruloplasmin). This sequestration of metal ions is an important antioxidant defense. Oxidative stress can provide iron for free radical reactions.

$$H_2O_2 + Fe^{2+} \rightarrow OH + OH^- + Fe^{3+}$$

B-ENZYME DEFENCE:

Living organisms have evolved antioxidant defenses to remove excess superoxide by accelerating its conversion to hydrogen peroxide. Superoxide dismutases (SODs) remove superoxide by accelerating its conversion to hydrogen peroxide. Mammalian cells have superoxide enzyme manganese (MnSOD) and its active site is mitochondria. A superoxide dismutase with copper and zinc (CuZnSOD) is largely present in the cytosol. It has been recently shown that familial dominant form of amylotrophic lateral sclerosis, a fatal degenerative disorder of motor neurons in the brain and spinal cord is related to mutations in the CuZnSOD. Hydrogen Peroxide generated by superoxide dismutase is converted to water and oxygen by enzyme catalase.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Catalases are present in the peroxisomes of mammalian cells, and serve to destroy hydrogen peroxide generated by oxidase enzymes located within these subcellular organelles. Glutathione is another enzyme which helps in removing hydrogen peroxide by converting it to water. This enzyme converts hydrogen peroxide to water by using it to oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG). Catalase and glutathione peroxides are enzymes whose role is to safely decompose peroxides. Catalase is present in the peroxisome and glutathione is present in cytosol of most cells and is active towards hydrogen peroxide.

1.7.3 Types of Antioxidants

1.7.3.1 Endogenous Antioxidants

Glutathione is tripeptide of glutamate, cysteine and glycine. It is found at a high (millimolar) concentration in most aerobic cells. As an antioxidant, glutathione plays a critical role in reducing hydrogen peroxide to water by enzyme glutathione peroxidase.

Alpha-tocopherol (Vitamin E) is the most important free radical scavenger within membranes and lipoproteins. Alpha tocopherol inhibits lipid peroxidation by scavenging peroxy radicals which are intermediates in the chain reactions.

$$LOO + \alpha\text{- tocopherol-OH} \longrightarrow LOOH + \alpha\text{- tocopherol-O}$$

Though the resultant alpha tocopheroxyl radical is free it is relatively stable. It is less effective at abstracting hydrogen when compared to the peroxy radicals, thereby slowing the chain reactions. Deprivation of alpha tocopherol in rats has resulted in severe neurological disorders confirming its role as a lipophilic antioxidant.

Ascorbic acid, the water soluble vitamin C is a good scavenger of several reactive oxygen species. It is essential in recycling of alpha-tocopherol from tocopheroxyl radical.

1.7.3.2 Exogenous Antioxidants

Endogenous anti-oxidant defence systems scavenge and minimize the formation of oxygen free radicals. However in pathological conditions demanding exogenous antioxidants, large number of drugs and natural compounds have been studied and characterized as potential antioxidants. These are not produced by the human body but may protect against pro-oxidant forces when administered as supplement (Sen, 1995). A large series of anti-inflammatory drugs have been recently tested with regard to their iron-binding and hydroxyl radical scavenging actions, and were found to be protective against site-specific damage by the hydroxyl radical. NSAIDs act not only by inhibiting cyclooxygenase, but also by stimulating SOD. Such an effect has been demonstrated for indomethacin, piroxicam and acetyl salicylic acid. Nimesulide have also been shown to inhibit the respiratory burst of phagocytes.

Carotenoid pigments, such as beta carotene, are powerful quenchers of singlet oxygen and found to have therapeutic value.

1.7.4 Antioxidants used in our study

1.7.4.1 Alpha Lipoic Acid (ALA)

Alpha lipoic acid, also known as lipoic acid or thioctic acid was discovered in 1951 as a molecule that assists in acyl-group transfer and as a coenzyme in the Krebs cycle. In 1980's the scientific community realized it is a powerful, natural antioxidant slowly becoming recognized as having some unique properties in the therapy and prevention of a broad range of diseases. Several qualities distinguish alpha-lipoic acid from other antioxidants: ALA can be synthesized by animals and humans ;(Carreau, 1979). It neutralizes free radicals in both the fatty and watery regions of cells, in contrast to vitamin C (water soluble) and vitamin E (fat soluble); and ALA functions as an antioxidant in both its reduced and oxidized forms (Packer et al., 1995).

Alpha lipoic acid also known as thioctic acid is a disulfide compound that is a cofactor in vital energy producing reactions in the body. It is also a potent biological antioxidant. Alpha lipoic acid was once thought to be a vitamin for animal and human .it is made endogenously in humans-the details of its synthesis are still not fully understood. Resent research indicates that the antioxidant role of alpha lipoic acid may confer several health benefits. Alpha lipoic acid contain a chiral center and consists of two enantiomers, the natural R or D enantiomer and the S or L enantiomer.commertial preparation of Alpha lipoic acid consists of the racemic mixture i.e. a50/50 mixture of R and E enantiomer.alpha lipoic acid has a variety of names. It is also known as lipoic acid, thiocitic acid, 1, 2-dithiolane-3-pentanoic delta-[3-(1, 2-dithiacyclopentyl)] pentanoic acid; acetate replacing factor and pyruvate oxidation factor.

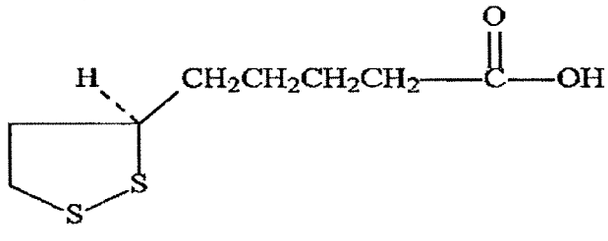


Fig 8: Alpha Lipoic Acid

Alpha lipoic acid has solubility in both aqueous and fat region of the body hence it is often termed as "**Universal antioxidant**".

Pharmacokinetics of ALA:-

ALA appears to be readily absorbed from an oral dose and converts easily to its reduced form, dihydrolipoic acid (DHLA), in many tissues of the body).The effects of ALA and DHLA are present both intra and extracellularly. ALA contains an asymmetrical carbon and thus has two possible optical isomers. These are designated as R-lipoic acid (R-ALA) and S-lipoic acid (S-ALA). Naturally occurring ALA is in the R configuration, bound to a protein where it functions as an essential cofactor for several mitochondrial enzyme complexes involved in energy production and in the catabolism of alpha-keto acids and amino acids (Bustamante et al., 1998).

Mechanism of Action:-

Alpha-lipoic acid is a potent antioxidant in both fat and water soluble mediums. Furthermore, its antioxidant activity extends to both its oxidized and reduced forms. DHLA is capable of directly regenerating ascorbic acid from dehydroascorbic acid and indirectly regenerating vitamin E (Scholich et al., 1989). Researches have also found ALA increases intracellular glutathione (Busse et al., 1992) and coenzyme (Kagan et al., 1990) levels.

Alpha-lipoic acid appears capable of chelating certain metals. It forms stable complexes with copper, manganese, and zinc (Sigel et al., 1978). In animal studies, it has been found to protect against arsenic poisoning (Grunert, 1960) and in both animal and in vitro studies, ALA reduced cadmium-induced hepatotoxicity (Muller and Menzel, 1990). In vitro, ALA chelated mercury from renal slices (Keith et al., 1997).

Clinical Indications:-

Diabetes: Mechanisms that may account for lipoic acid's benefit in preventing diabetic complications include prevention of protein glycosylation (Schleicher, 1997) and inhibition of the enzyme aldose reductase, the latter of which subsequently inhibits conversion of glucose and galactose to sorbitol (Kishi et al., 1999). Accumulation of sorbitol has been implicated in the pathogenesis of various diabetic complications, including "sugar cataracts" where sorbitol accumulates in the lens. Lipoic acid has the potential to prevent diabetes (at

least in animals), influence glucose control, and prevent chronic hyperglycemia-associated complications such as neuropathy. Acting as a potent antioxidant, DHLA protected rat pancreatic islet cells from destruction by reactive oxygen species (Heller et al., 1997). In vitro, lipoic acid stimulated glucose uptake by muscle cells in a manner similar to insulin (Estrada et al., 1996). Type 2 diabetics given 1,000 mg lipoic acid intravenously (i.v) experienced a 50-percent improvement in insulin-stimulated glucose uptake (Jacob et al., 1995). In an uncontrolled pilot study, 20 Type 2 diabetics were given 500 mg lipoic acid i.v for 10 days. While there was an average 30-percent increased uptake of glucose, there were no changes in fasting sugar or insulin levels (Jacob et al., 1996). In a study examining the effect of lipoic acid as a cofactor of the pyruvate dehydrogenase complex on both lean and obese Type 2 diabetics, insulin sensitivity, glucose effectiveness, serum lactate levels, and pyruvate levels were tested after oral glucose tolerance load. Treatment with 600mg ALA twice daily for four weeks increased insulin sensitivity and prevented serum lactate/pyruvate-induced hyperglycemia (Konrad et al., 1999). In a placebo-controlled, multi-center pilot study, 74 patients with Type 2 diabetes were randomized to receive 600mg ALA or placebo orally once, twice, or three times daily, for four weeks. Although no significant differences regarding the various doses of ALA were observed, at the end of the trial it was found that patients who received ALA experienced significant improvement in insulin-stimulated glucose disposal compared to those of placebo (+27%; $p<0.01$). This suggests that oral administration of ALA can improve insulin sensitivity in Type 2 diabetics (Jacob et al., 1999). Experimental research indicates R-ALA may be more effective than S-ALA in improving insulin sensitivity. In an animal model of non-diabetic insulin resistance, R-ALA for 10 days increased glucose uptake by skeletal muscle of obese rats by 65 percent, compared to 29 percent with S-ALA. In addition, R-ALA significantly reduced plasma insulin by 17 percent; whereas, S-ALA increased insulin levels by 15 percent. This seems to indicate an increase in insulin resistance with S-ALA (Streeper et al., 1997).

Diabetic Nephropathy: One animal study suggested that ALA may be effective in the prevention of early diabetic glomerular injury and may provide more protection than high doses of vitamin C or vitamin E (Melhem et al., 2001). The study observed ALA (30mg/kg body weight daily for two months) given to diabetic rats either prevented or significantly attenuated increases in urinary albumin excretion, fractional albumin clearance, glomerular

volume, and glomerular content of immunoreactive transforming growth factor-β and collagen α addition, it was found that ALA, but not vitamins C or E, significantly increased renal-cortical glutathione content.

Diabetic Neuropathy: Alpha-lipoic acid has been studied extensively in Europe for the treatment of diabetic neuropathy (Ziegler et al., 1999). Three large-scale, double blind, placebo-controlled trials have been conducted on the effect of ALA for neuropathy- the Alpha-Lipoic Acid in Diabetic Neuropathy (ALADIN) studies. The first ALADIN study (n=328 Type 2 diabetics) found three weeks of ALA at 600mg daily was superior to placebo for reducing the symptoms of neuropathy (Ziegler et al., 1995). ALADIN II examined nerve conduction parameters in a two-year trial and found improvement in some nerve conduction parameters with ALA compared to placebo (Reljanovic et al., 1999). In the seven-month ALADIN III trial, 509 subjects received either 600 mg orally three times daily for six months or 600 mg I.V ALA daily for three weeks, followed by placebo three times daily for six months; or double placebo. While no significant differences were noted in subjective symptom evaluation among the groups, treatment with ALA was associated with improvement in nerve function (Ziegler et al., 1999). In one randomized, double-blind, placebo-controlled study, known as the SYDNEY trial, 60 Type 2 diabetic (DSPN) were administered 600 mg ALA I.V daily, five days/week for a total of 14 treatments, while an equivalent number of DSPN patients were given placebo for the same duration. At the end of the trial, overall symptoms (e.g., lancinating and burning pain, numbness and tingling) compared to the placebo group (average decrease in total symptom score (TSS): 5.7 versus 1.8) ($p<0.001$) (Ametov et al., 2003). Another smaller study demonstrated similar results using oral ALA. In this trial, 24 Type 2 diabetic patients with symptomatic polyneuropathy were randomly assigned to 600 mg ALA three times daily or placebo for three weeks. Neuropathic symptoms (pain, burning, paresthesia, and numbness) in the feet were scored regularly and summarized as a TSS. At the end of the trial the ALA group reported significant improvements in TSS compared to placebo (TSS decreases: 3.8 versus 1.9) ($p=0.021$) (Ruhnau et al., 1999). Lipid peroxidation is believed to play a role in the development of neuropathy. In an in vitro study, lipoic acid decreased lipid peroxidation of nerve tissue (Nickander et al., 1996). ALA may also be beneficial for diabetic cardiac

autonomic neuropathy (CAN). A four month trial of oral ALA at a dose of 800 mg daily (n=39) or placebo (n=34) demonstrated a trend toward improvement (Ziegler et al., 1997)

Cataract: The enzyme aldose reductase plays an important role in the development of cataracts in diabetics. Lipoic acid inhibited aldose reductase activity in the rat lens. In another animal study, ALA inhibited cataract formation experimentally induced by buthionine sulfoximine, an inhibitor of glutathione synthesis. ALA administration maintained levels of glutathione, ascorbic acid, and alpha-tocopherol in the lens (Maitra et al., 1995). These same researchers found that R-ALA reached concentrations in rat lens two to seven fold higher than S-ALA, with the racemic mixture reaching levels between the two (Maitra et al., 1996).

Glaucoma: Lipoic acid was administered to 75 subjects with open-angle glaucoma at dosages of either 75 mg daily for two months or 150 mg daily for one month. Thirty-one others served as controls and were given only local hypotensive therapy. The greatest improvement in the biochemical parameters of glaucoma and visual function were observed in the group receiving 150 mg lipoic acid (Filina et al., 1995).

Ischemia-Reperfusion injury: After an area of tissue has been deprived of blood for a period of time, such as occurs in the brain after a stroke or in the heart after clot dissolution, reperfusion of the tissues causes a burst of free radical formation. Several animal studies have demonstrated the effectiveness of DHLA in the prevention of reperfusion injury (Prehn et al., 1992) (Cao and Phillis, 1995).

Alcoholic liver disease: Although preliminary studies have indicated possible benefit of lipoic acid in the treatment of alcoholic liver disease, the only controlled, double-blind study found ALA had no significant influence on the course of the disease (Marshall et al., 1982).

Cognitive function: Alpha-lipoic acid may have a positive effect on patients with Alzheimer's disease and other types of memory dysfunction secondary to trauma or cerebral vascular accident. By decreasing oxidative damage in the central nervous system, ALA may decrease the severity of central nervous system disorders (Packer et al., 1995). An animal study has shown supplementation with lipoic acid improves long-term memory in aged mice; however, no effect in young mice was shown (Stoll et al., 1993). This lack of treatment effect in young mice suggests ALA does not improve memory from a general standpoint; instead, it

appears ALA compensates for age-related memory deficits. alpha-lipoic also appears to protect brain cells from damaging effects of some hazardous chemicals.

Heavy metal toxicity: In vitro and animal studies suggest lipoic acid supplementation might be a beneficial component in the treatment of heavy metal toxicity, particularly toxicity involving lead, cadmium, mercury, or copper (Gurer et al., 1999; Muller and Menzel, 1990; Muller, 1989; Sumathi et al., 1996). In one study an intraperitoneal injection of 25 mg/kg ALA given to rats for seven days was able to significantly alter the oxidative stress induced by lead toxicity. (Gurer et al., 1999). Another study demonstrated ALA at concentration of 5mM, was able to protect rat hepatocytes from cadmium toxicity when cadmium was given at the concentration of 200 mM (by preventing decreases in total glutathione and increases in lipid peroxidation (Muller and Menzel, 1990). Furthermore, a study of mercury intoxication revealed an injection of 10mg/kg/day ALA in rats inoculated with 1 mg/kg/day mercuric chloride prevented damage to nerve tissue caused by lipid peroxidation (Anuradha and Varalakshmi, 1999). Long-Evans Cinnamon rats have a genetic defect that causes them to accumulate copper in the liver in a manner similar to patients with Wilson's disease and spontaneously develop acute hepatitis. ALA has been shown to protect these rats from developing hepatitis (Yamamoto et al., 2001). ALA appears to improve tissue redox status in metal toxicity and during chelation with dithiol compounds, including dimercaptosuccinic acid (DMSA) (Pande and Flora, 2002). Anecdotal reports note the use of lipoic acid may improve the clearance of toxic metals.

Other indications: Other therapeutic uses for ALA or DHLA include protection from radiation injury and prevention of HIV viral replication by inhibition of reverse transcriptase and nuclear factor kappa-B (a protein that functions as a nuclear transcription factor and appears to play a role in inflammation) (Baur et al., 1991).

1.7.4.2 Coenzyme Q10:

CoQ10 is a member of quinone family, composed of a 'quinone'' nucleus in which two of the four hydrogen atoms are each replaced by a methoxy group (Meo), one by a methyl group (Me) and the fourth is replaced by a chain of ten isoprene units. This is why it is designated as Q10.

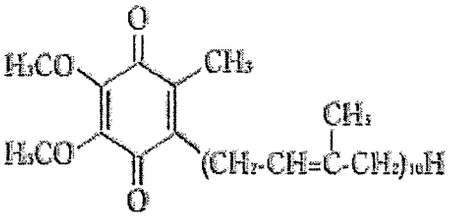


Fig 9: Chemical structure of CoQ10

CoenzymeQ10 (CoQ10), also known as ubiquinone, a name that signifies its ubiquitous (widespread) distribution in the human body is an endogenously synthesized antioxidant. It is a component of the oxidative phosphorylation in the mitochondria, which converts the energy in carbohydrates and fatty acids into ATP to drive cellular machinery (Ernsteer and Dallner, 1995). CoQ10 is thus involved in the energy producing mechanism in the cell called Krebs cycle, the mechanism in which the body produces ATP or fuel without which the cell and the human body cannot surview. CoQ10 is thus called the human LIFE SPARK. CoQ10 is found in abundance in the heart, liver, kidneys and brain, the hardest working organ in the body, in the mitochondria.

Table Distribution of CoQ1O in Human Organs (mg/g ww)...

Age	Heart	Kidney	Lung	Spleen	Liver	Pancreas	Adrenal
1 - 3 days	36	17	2	20	12	9	17
0.7- 2 yrs	78	53	6	30	45	38	57
19-21 yrs	110	98	6	32	61	21	16
39- 43 yrs	75	71	6	28	58	19	12
77-81 yrs	47	64	3	13	50	6	8

The biosynthesis of CoQ10 from the amino acid tyrosine is a multistage process requiring atleast eight vitamins and several trace elements. In the related field of free radical chemistry, CoQ10 has been studied in its reduced form as a potent antioxidant.

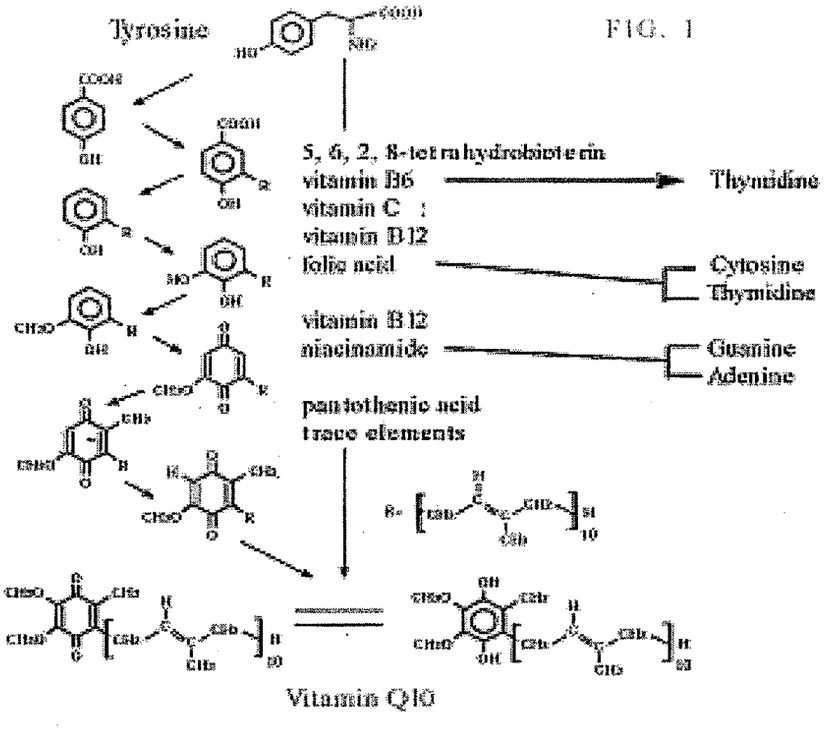


Figure 10: Biosynthesis of CoQ10

1.7.4.2.1 Clinical uses of CoQ10

In Diabetes

In diabetes, mitochondrial oxidative phosphorylation is significantly reduced; thus ATP production is reduced along with decreased level of CoQ10. In addition to assisting electron transfer during oxidative phosphorylation, CoQ10 inhibits certain enzymes involved in the formation of free radicals and thus attenuates oxidative stress. It can also function beneficially by virtue of its free radical scavenging and inhibition of phospholipase activity. (Stocker and Frei, 1991). Chronic CoQ10 treatment significantly decreases serum cholesterol and triglyceride levels in fructose fed animals, indicating improvement in lipid metabolism.

Singh et al. reported the reduction in coronary artery plaque size, atherosclerotic index, cholesterol and triglyceride in Coenzyme Q10 treated transgenic diet fed animals (Singh et al.,2000) FFA and glucose overload increases the generation of acetyl CoA which in turn increases the production of electron donors from the tricarboxylic acid cycle. This increases the membrane potential, because protons are pumped across the mitochondrial inner membrane in proportion to electron flux through the electron transport chain. Inhibition of electron transport at complex III by increased membrane potential increases the half-life of free radical intermediates of CoQ10 which reduces O_2 to superoxide (Shin et al., 2001). According to an intraperitoneal glucose tolerance test, treatment with antioxidants retained glucose stimulated insulin secretion and moderately decreased the blood glucose levels (Yoshitaka and Hideaki, 2004). CoQ10 treatment reduced the blood glucose levels in fructose fed animals without affecting hyperinsulinemia. CoQ10 treatment also significantly decreased lipid peroxidation and increased SOD, catalase and reduced glutathione levels in the liver tissue homogenate, which might be the result of a decrease in oxidative stress. In coronary artery disease patients, CoQ10 supplements were also associated with a significant reduction in thiobarbituric acid reactive substances, malondialdehyde and diene conjugates (Singh et al., 1999). In conclusion CoQ10 a provitamin and a mitochondrial free radical scavenger was found to significantly improve deranged carbohydrate and lipid metabolism of experimental fructose diet induced insulin resistant state in rats. The mechanism of its in-vivo antihyperlipidemic and antidiabetic action appears to be its mitochondrial antioxidant activity.

In apoptosis

Coenzyme Q10 is also known to prevent apoptosis. The permeability transition pore (PTP) is a mitochondrial channel whose opening causes mitochondrial membrane potential collapse that leads to apoptosis. Some ubiquinone analogs have been previously demonstrated to modulate the PTP open-closed transition in isolated mitochondria and thought to act through a common PTP binding site rather than through oxidation-reduction reactions. It has been demonstrated both in vitro and in vivo that the ubiquitous free radical scavenger and respiratory chain Coenzyme Q10 prevents keratocyte apoptosis induced by excimer laser irradiation more efficiently than other antioxidants.

Infertility: Synthesis of sperm requires considerable energy. CoQ10 is used by the body to transform food into the energy on which the body runs i.e.ATP. Preliminary research reports that supplementation of CoQ7, a related molecule, increased sperm counts in a group of infertile men (Tanimura, 1967)

Gum diseases: Healing of periodontal tissue (the gums of the mouth) may require increased energy production; therefore, researchers have explored the effects of CoQ10 supplementation in people with periodontal disease which has been linked to CoQ10 deficiency. Double-blind research shows that people with gum disease given CoQ10 achieve better results than those given a placebo (Gaby, 1998)

Angina: Patients taking 150 mg per day of CoQ10 report a greater ability to exercise without experiencing chest pain. (Kamikawa et al., 1985).

Alzheimer's disease: Mitochondrial function also appears to be impaired in people with Alzheimer's disease. Due to CoQ10's effects on mitochondrial functioning, one group of researchers has given CoQ10 (along with iron and vitamin B6) to several people with Alzheimer's disease and reported the progression of the disease appeared to have been prevented for one and a half to two years (Imagawa et al., 1992)

Breast cancer: CoQ10 appears to modulate immunity. Perhaps as a result, a few cases have been reported in which women with metastatic breast cancer had a regression of their cancer after treatment with a very large amount CoQ10 (390 mg per day) (Lockwood et al., 1995).

Hypertension: CoQ10 appears to modulate blood pressure by reducing resistance to blood flow. Several trials have reported that supplementation with CoQ10 significantly reduces blood pressure in people with hypertension, usually after ten weeks to four or more months of treatment (Gaby, 1996). CoQ10 has been utilized to treat hypertension. The department of medicine, Mt. Sinai Hospital and Medical Center in New York, reported in the Journal of Clinical Pharmacology that its cardiovascular importance is now being realized in clinical trials worldwide. (Greenberg and Frishman, 1990) In humans, a deficiency of CoQ10 was found in 39% of patients with hypertension, compared to 6% to those with normal blood pressure. Providing these patients with 60 mg of CoQ10 for eight weeks resulted in a 10% or

greater decrease in blood pressure (Yamagami et al., 1975). In a double blind study, 20 hypertensive subjects with low serum CoQ10 levels receiving 100 mg of CoQ10 per day for 12 weeks, showed a significant reduction in systolic and diastolic blood pressure (Yamagami et al., 1986). In a 1994 study, 109 patients with known hypertension were given 225 mg of CoQ10 daily, achieving a serum level of at least 2mcg/ml. There was a decrease in systolic blood pressure from an average of 159mm Hg to 147mm Hg, while mean diastolic pressures dropped from 94 to 85 mm Hg. Fifty percent of patients were able to decrease or eliminate their medication (Langsjoen et al., 1994). The mechanism by which CoQ10 reduces blood pressure is not fully understood. However in 1990, Digiesi and Cantini demonstrated a decrease in the resistance of blood vessels (Digiesi and Cantini, 1990).Further, clinical cardiologist Stephen Sinatra, MD, FACC, believes this action may be secondary to an improvement in the metabolic function of the cells, and that the antioxidant properties of CoQ10 may help normalize cellular chemistry and promote optimal tone and compliance of the elastic vessel walls (Sinatra and Steven, 1998).

Myopathy- Q10 is involved in development in statin myopathy (1) HMG-CoA reeducates inhibitor used to treat elevated blood cholesterol level by blocking cholesterol biosynthesis and also block CoQ10 biosynthesis. Supplementation with CoQ10 in statin myopathy improves the condition.

Cancer:- Low level of Q10 in the blood of some cancer patient have been noted.CoQ10 causes significant reduction in the cardiac toxicity of the chemotherapeutic drug adryamicin.The cardiac toxicity of adriamycin may be related to free radical generation and this might explain the benefit of CoQ10 in its capacity as a free radical scavenger.

AIDS: - Another interested topic is the relationship between the immune system and CoQ10.End stage of AIDS has been associated with a significant deficiency of Q10.

Parkinson's disease: - Q10 might be effective in the prevention and treatment of parkinse disease. A study showed that the brain cells of Parkinson's specific impairment that causes the destruction of healthy mitochondrial function. It is known that mitochondrial disorder

causes nigra region of the brain to multifunction and died, thus creating the shortage of Dopamine.All metabolically active tissues are highly sensitive to a deficiency of CoQ10.Diverse number of disease states responds favorably to Q10 supplementation.

1.7.4.3 Selenium

Selenium was discovered in 1818 by a Swedish chemist Jons Jacob Berzelius. He named it Selene after the Greek goddess of the moon. One hundred and forty years later, Schwartz and Foltz identified selenium as essential to animal health when they discovered that trace amounts protected against liver necrosis in vitamin E deficient rats (Schwartz and Foltz, 1957). Interest in the role of selenium in human health gathered momentum in the late 1960's, and investigations looked for human diseases similar to those of Se- responsive animal disorders. (Combs and Combs, 1986). Although selenium was identified as essential to human nutrition 42 years ago, a universal marker of daily requirement remains elusive. Research has extended our knowledge of the essential functional roles attributed to selenium, which have both short and long term public health implications. Selenium is a potent antioxidant. Elemental selenium is practically inert nutritionally and toxicologically. On the other hand, different inorganic or organic compounds of selenium can be converted in a mammalian organism into biologically active form. It is an integral part of the body's natural antioxidant-glutathione peroxidase system, and at times in partnership with Vitamin A protects against cancer and prevents lipid peroxidation. Selenium may influence the synthesis of glutathione peroxidase, which facilitates the lowering of tissue peroxide levels in the body by destroying hydrogen peroxide. Levels of selenium compounds in the food necessary for the prevention of selenium deficiency are dependent on dietary levels of vitamin E. There is overlap in the action of selenium and vitamin E in that both are responsible for lowering tissue peroxide levels. It is also an effective detoxifier of heavy metals, its antioxidant properties protects against environmental and chemical sensitivities, and its immune function enhances the body's antibacterial and antiviral defenses. Selenium is present in soil and enters the food chain through plants. We obtain most of our dietary Se from bread, cereal, meat and poultry. Tissue levels of Se are readily influenced by the dietary intake which itself is goverened by geographical differences in available selenium in soil. In general, soil concentration of available Se is low in Europe. Bioavailability of Se is also low and has

decreased further as a consequence of increasing acid rain and use of excessive fertilization. Generally speaking, human blood Se levels follow the same geographical pattern around the world as those of livestock in the same regions (Casey et al., 1982). Although Se deficiency in animals has long been recognized, obvious clinical signs of human Se deficiency are rare. The exception is in areas of North-East China with very low soil Se where an endemic, fatal cardiomyopathy, Keshan disease was found to respond to Se supplementation. The best source of Selenium is seafood.

1.7.4.3.1 Clinical uses of Selenium

Cancer

There is strong evidence that Se has a protective effect against some forms of cancer. In a recent study involving 1312 patients supplemented with 200 μg Se daily, the incidence of prostate, colon and lung cancers was decreased by 63, 58 and 46% respectively (Clark et al., 1996). The mechanism of action of the chemoprotective effects are not known by may be mediated through the two major Se dependent redox systems in the cell. Several studies have suggested a possible link between cancer and selenium deficiency (Russo et al., 1997; Knekt et al., 1998; Young and Lee, 1999; Burguera et al., 1990). One study, known as the NPC (National Prevention of Cancer), was conducted to test the effect of selenium supplementation on the recurrence of skin cancers on selenium-deficient men. It did not demonstrate a reduced rate of recurrence of skin cancers, but did show a reduced occurrence of total cancers, although without a statistically significant change in overall mortality(Clark et al.,1996).The preventative effect observed in the NPC was greatest in those with the lowest baseline selenium levels(Lippman et al.,2009).In 2009 the 5.5 year SELECT study reported that selenium and vitamin E supplementation, both alone and together, did not significantly reduce the incidence of prostate cancer in 35,000 men who "generally were replete in selenium at baseline"(Lippman et al.,2009).The SELECT trial found that vitamin E did not reduce prostate cancer as it had in the Alpha-Tocopherol, Beta Carotene (ATBC) study,but the ATBC had a large percentage of smokers while the SELECT trial did not(Lippman et al.,2009).

Dietary selenium prevents chemically induced carcinogenesis in many rodent studies(Chemoprevention Database,2009).It has been proposed that selenium may help prevent cancer by acting as an antioxidant or by enhancing immune activity.

Not all studies agree on the cancer-fighting effects of selenium.One study of naturally occurring levels of selenium in over 60,000 participants did not show a significant correlation between those levels and cancer(Garland et al.,1995). The SU.VI.MAX study (Hercberg et al., 1998) concluded that low-dose supplementation (with 120 mg of ascorbic acid, 30 mg of vitamin E, 6 mg of beta carotene, 100 μg of selenium, and 20 mg of zinc) resulted in a 30% reduction in the incidence of cancer and a 37% reduction in all-cause mortality in males, but did not get a significant result for females (Hercberg et al., 2004). However, there is evidence that selenium can help chemotherapy treatment by enhancing the efficacy of the treatment, reducing the toxicity of chemotherapeutic drugs, and preventing the body's resistance to the drugs(Selenium and Chemotherapy).Studies of cancer cells in vitro showed that chemotherapeutic drugs, such as Taxol and Adriamycin, were more toxic to strains of cancer cells grown in culture when selenium was added(Selenium Cancer;Nilsonne et al., 2006).

In March 2009, a study from the Department of Cancer Biology at the University of Texas M. D. Anderson Cancer Center reports that Vitamin E (400 IU) and selenium (200 micrograms) supplements affect gene expression and can act as a tumor suppressor(Tsarachidou et al., 2009). Eric Klein, MD from the Glickman Urological and Kidney Institute in Ohio said the new study "lend credence to the previous evidence that selenium and vitamin E might be active as cancer preventatives"(Klein, 2009). In an attempt to rationalise the differences between epidemiological and *in vitro* studies and randomised trials like SELECT, Klein said that randomized controlled trials "do not always validate what we believe biology indicates and that our model systems are imperfect measures of clinical outcomes in the real world"(Klein, 2009).

HIV/ AIDS

Some research has indicated a geographical link between regions of selenium-deficient soils and peak incidences of HIV/AIDS infection. For example, much of sub-Saharan Africa is low in selenium. However, Senegal is not, and also has a significantly lower level of AIDS infection than the rest of the continent. AIDS appears to involve a slow and progressive decline in levels of selenium in the body. Whether this decline in selenium levels is a direct result of the replication of HIV(Patrick,1999) or related more generally to the overall malabsorption of nutrients by AIDS patients remains debated.

Low selenium levels in AIDS patients have been directly correlated with decreased immune cell count and increased disease progression and risk of death(Dietary Supplement Fact Sheet:Selenium 2009). Selenium normally acts as an antioxidant, so low levels of it may increase oxidative stress on the immune system leading to more rapid decline of the immune system. Others have argued that HIV encodes for the human selenoenzyme glutathione peroxidase, which depletes the victim's selenium levels. Depleted selenium levels in turn lead to a decline in CD4 helper T-cells, further weakening the immune system(Taylor, 1995).

Regardless of the cause of depleted selenium levels in AIDS patients, studies have shown that selenium deficiency does strongly correlate with the progression of the disease and the risk of death(Baum et al., 1997;Campa et al., 1999;Baum and Shor-Posner, 1998).

Tuberculosis

Some research has suggested that selenium supplementation, along with other nutrients, can help prevent the recurrence of tuberculosis(Villamor et al., 2008).

Diabetes

A well-controlled study showed that selenium intake is positively correlated with the risk of developing Type II diabetes. Because high serum selenium levels are positively associated with the prevalence of diabetes, and because selenium deficiency is rare, supplementation is not recommended in well-nourished populations such as the U.S(Bleys and Joachim, 2007).

Mercury toxicity

Experimental findings have demonstrated a protective effect of selenium on methylmercury toxicity, but epidemiological studies have been inconclusive in linking selenium to protection against the adverse effects of methylmercury(Watanabe, 2002).

Cardiovascular disease

Low blood selenium concentrations have been associated with increased cardiovascular disease mortality. This may be a reflection of sub-optimal GPx4 activity in the prevention of LDL oxidation, with subsequent uptake by endothelial cells and macrophages in arterial blood vessels. Heart disease mortality declined by an average 61% in Finland between 1972 and 1992. The decline has been attributed to major lifestyle changes, the most important reported as a 4% fall in energy consumed from fat, with an associated lowering of blood cholesterol concentration. It is highly likely, that the concomitant 3 fold increase in dietary selenium intake as a consequence of selenium enrichment of fertilizer, since 1985 would also have contributed to lower heart disease mortality reported in 1992.

Immune function

Although the mechanisms involved have yet to be fully elucidated, it is well established that dietary selenium is important for a healthy immune response (Taylor,1995). Dietary supplementation of humans with 200μg of sodium selenite enhances T-lymphocyte immune responses (Roy et al., 1994). A progressive decline in plasma Se has been widely reported in adult respiratory distress syndrome (ARDS) and AIDS patients, and approximately parallels T-cell loss or stage of HIV infection. It is particularly noticeable at the terminal stage of disease where Se deficiency is now considered a classical symptom/sign of end stage. There is an extremely high turnover of CD4 T-cells in AIDS, with billions of new cells lost and replaced daily. The constant formation of new cells to replace those lost requires an extremely efficient and effective Se supply to keep up with the high demand in active lymphocytes.

Asthma

Functional selenium and vitamin E status may influence leucotriene metabolism and has important implications in relation to chronic inflammatory disease, particularly asthma which is now the most prevalent chronic inflammatory condition in childhood, and has doubled over the last 20 years in the UK (Seaton et al., 1994). There is a dramatic rise in the prevalence of asthma in the UK which mirrors the dramatic decline in blood Se concentration.