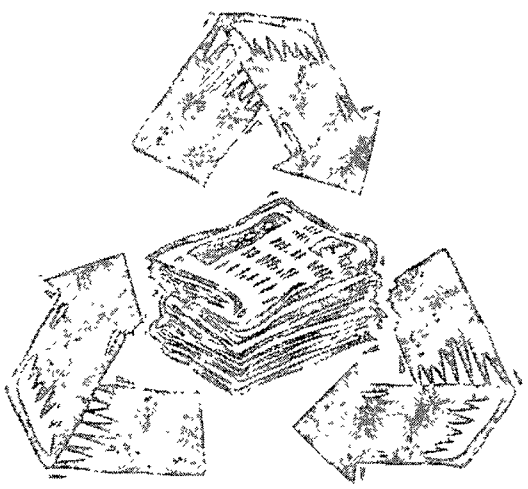
CHAPTER – 2



INTRODUCTION

2.1 OXIDATIVE STRESS

In the late 1950's, free radicals and antioxidants were almost unheard of in the clinical and biological sciences but chemists had known about them for years in the context of radiation, polymer and combustion technology. In early 1960's, there was first evidence of the toxic effects ionizing radiation on elevated oxygen levels in aerobes and it was proposed that oxygen toxicity is due to free radical formation (Gutteridge and Halliwell, 2000). By the 1980's, molecular biology had been evaluated from biochemistry and microbiology and became a dominant new discipline. As a biological tool to explore reaction mechanisms, superoxide dismutase was a unique and valuable asset. Its ability to inhibit radical reactions leading to oxidative damage *in vitro* often turned out to be due to its ability to prevent reduction of iron ions by superoxide. After molecular biology, pharmacology and therapeutics became active for finding the molecules for quenching free radicals. The 1990's could reasonably be deemed the Antioxidant Decade.

Exposure to reactive oxygen intermediates or free radicals cause oxidative stress. 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, such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) that can damage proteins, nucleic acids and cell membranes. Increasing evidence suggests that the cumulative damage caused by reactive oxygen species contribute to numerous diseases. (Aruoma and Halliwell, 1998). Recent studies also suggest that the effects of these oxidants are integrally linked to the damage caused by hypochlorous acid (HOCl) and the reactive nitrogen intermediates nitric oxide (NO), peroxynitrite (HOONO) and nitrosothiols (RSNO).

Normally a balance between oxidative events and antioxidative forces maintains the *status quo* within living cells. A variety of enzymes help to maintain in a reduced state despite the presence of aerobic environment. Thus, major cellular reducing agents, such as vitamin E, vitamin C, and glutathione are present predominantly in their reduced forms. In addition, a number of enzymes scavenge and remove these reactive chemical species. When 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 oxidative stress*.

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.

With the exception of unusual circumstances such as the influence of ionizing radiation, free radicals are generally produced in cell by electron transfer reactions. These can be mediated by the action of enzymes or non-enzymatically, often through the redox chemistry of transition metal ions.

Under normal circumstances, the major source of free radicals in cells is electron 'leakage' from electron transport chains, such as those in mitochondria and in the endoplasmic reticulum, to molecular oxygen, generating sueproxide. Other enzymes can also produce superoxide or hydrogen peroxide, such as the range of flavin oxidases located in peroxisomes. Another source of superoxide in animal cell is the autoxidation of certain compounds including ascorbic acid (vitamin C), thiols (e.g. glutathione, cysteine), adrenaline and flavin co-enzymes. These autoxidation reactions can be greatly enhanced by the involvement of transition metal ions. This accidental production of free radicals is kept to a minimum by the high efficiency of enzyme-mediated electron transfer and by keeping metal ions tightly sequestered; these are the fundamental means of preventive antioxidant defense.

With the increasing acceptance of free radicals as commonplace and important biochemical intermediates, they have been implicated in a very large number of human diseases. Consequently, free radical ablation offers a substantial potential for the treatment 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 inflammation (Winrow et al., 1993), cancer (Guyton and Kensler, 1993), aging (Nohl, 1983), ischemia (Flitter, 1993), diseases of central nervous system (Evans, 1993), diabetes (Wolff, 1993), liver damage (Poli, 1993), atherosclerosis (Esterbauer et al., 1993), modification of LDL (Steinbrecher, 1987) have been published.

2.1.1 Lipid Peroxidation

Lipid peroxidation (LPO) is defined as the 'Oxidative deterioration of polyunsaturated fatty acid (Tappel, 1973). LPO is thought to be an important biological consequence of oxidative cellular damage (Plaa and Witschi, 1976). Lipid peroxidation is a destructive free radical mediated process for biological membranes, which has been implicated in a variety of disease state. 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. It has been associated with several physiological, pathological and toxic processes including prostaglandin biosynthesis (Fridovich, 1978), ageing (Hochstein and Jain, 1981) and many toxic reactions to various organs (Plaa and Witschi, 1976; Smith et al., 1983).

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, is mediated by the transition metal ions (Ottolenghi, 1959). Ions such as transition metals iron and copper are involved in many free radicals and their reactions. 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. The transferrine forms a complex with iron ions thereby reducing the iron dependent lipid peroxidation or hydroxyl radical formation. Uric acid can interfere with radical reaction not only by scavenging oxidants, but also by binding iron and copper ions in forms that do not participate in radical reaction (Halliwell and Gutteridge, 1989). Albumin is a sacrificial antioxidant. It inhibits copper dependent lipid peroxidation and hydroxyl radical formation. Ceruloplasmin is plasma copper containing protein, which catalyses the oxidation of Fe^{+2} ions. The ferroxidase action of ceruloplasmin allows the iron dependent lipid peroxidation or hydroxyl radical formation from hydrogen peroxide

(Halliwell and Gutteridge, 1989). However, oxidative stress can provide iron for free radical reactions. Thus, superoxide can mobilize iron from ferritin, although the amount of superoxide releasable iron is small (Simpson et al., 1992). Hydrogen peroxide can degrade heme protein to release iron (Cotran et al., 1989). Hence, a major determinant of the nature of the damage done by excess generation of reactive oxygen species *in vivo* may be the availability and location of metal in catalyzed hydroxyl radical formation.

2.1.2 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 pathogenesis of disease has led to an increased need to measure free radials and their reactions. The major problems in determination of most of the free radicals of interest that they are extremely reactive and possess very short life: if any produced *in vivo,* react at or close to their source of formation. Consequently, 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).

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.

The only analytical technique that directly measures the free radicals is electron spin resonance (ESR) spectrometry. However, since it is relatively insensitive and requires steady-state concentrations of free radicals in the micromolar range it is of very limited value for use *in vivo.* Whole-body ESR, analogous to whole -body NMR, has been investigated but not yet fully developed. Nevertheless, ESR has been used to detect free radicals in human tissue samples obtained *ex vivo*. ESR spectrometry can usually be applied to analysis of samples *in vivo* only through the technique of spin trapping by addition of a compound known as Spin-trap, which reacts free radicals to form radical-adducts. Spin traps have been used in experimental animals to demonstrate the production of free radicals *in vivo*, but no effective spin trap presently exist that can be administered in humans (Cheeseman and Slater, 1993).

An alternative technique for trapping hydroxyl radicals generated in the cells, organelles and perfused organs is by measuring the (non-radical) products formed from their attack on aromatic compounds. Thus, attack of hydroxyl radicals on salicylic acid produces 2, 3 dihydroxybenzoate and on phenylalanine produces *o*- and *m*-tyrosines (Grootved and Halliwell, 1986).

Lipid peroxidation is a complex process whereby polyunsaturated fatty acids (PUFAs) in the phospholipids of cellular membranes undergo reaction with oxygen to yield lipid hydroperoxides. The reaction occurs through a free radical chain mechanism initiated by the abstraction of hydrogen atom from a PUFA by a reactive free radical, followed by a complex sequence of propagative reactions. The hydroperoxide and conjugated dienes that are formed can decompose to form numerous other products including alkanals, alkenals, hydroxyalkenal, malondialdehyde (MDA) and volatile hydrocarbons (Halliwell and Gutteridge, 1989). Lipid peroxidation is often a first parameter in investigation of free radical injury.

Hydroperoxides are the major initial molecular products of lipid peroxidation and can be measured by a variety of reported methods. Hydroperoxides possess a conjugated diene structure having a characteristic UV absorption around 234 nm. Measurement of this absorbance has been extremely useful as an index of peroxidation in pure lipid systems and in tissue preparations from experimental animals.

The thiobarbituric acid (TBA) assay is the most popular and easiest method used as an indicator of lipid peroxidation and free radical activity in biological samples. The assay is based on the reaction of TBA with MDA, one of the aldehyde products of lipid peroxidation.

Apart from these aforementioned methods, there are several methods available for measurements of lipid peroxidation products like aldehydes (4-hydroxynonenol) and volatile hydrocarbons (ethane, pentane derived from hydroperoxides of ω-3 and ω-6-PUFAS respectively), and the measurement of fluorescent products of lipid peroxidation (dihydropyridine dicarbaldehydes).

The generation of free radicals is a continuous process; the pro-oxidants and other toxicants stimulate this process. Body has its own defense system against this continuous onslaught of free radicals in the form of some enzymes and cellular

compounds known as endogenous antioxidants. Superoxide dismutase, catalase and reduced glutathione are important amongst them. These endogenous antioxidants get exhausted in excessive generation of free radicals. The change in the levels of endogenous antioxidants is also related to the generation of free radicals. The methods for estimation of the levels of superoxide dismutase, catalase and reduced glutathione are well addressed in the literature. These estimations provide a sound base for confirming the release of free radicals.

The living cells contain a lipophilic cell membrane which is rich in lipids (PUFAs, cholesterol, triglycerides and phospholipids) and some membrane bound enzymes. The activities of the membrane bound enzymes (ATPases) largely depend on the lipids and electrolyte concentration. The free radicals are known to alter the lipids of the cell and affect the functioning of the cell by altering membrane fluidity and membrane bound enzymes (ATPases). Estimation of lipids are not directly indicate the free radical generation but give idea regarding lipid peroxidation, which is an indicator of free radical release. The estimation of lipids and membrane bound enzymes are well addressed in the literature. Such estimations certainly indicate about the functioning of the cell. Apart from lipids, the free radicals are also known to cause catabolism of the proteins, by modifying the amino acid residues and lead to cross-linking, changes in conformation and loss of function. Oxidatively damaged proteins are likely to be removed rapidly by protease rather than accumulate to readily detectable levels. There are various methods available for estimation of proteins in literature.

2.2 METAL IONS AND OXIDATIVE STRESS

Metal ions (mainly copper and iron) are known to catalyze the cleavage of hydrogen peroxide and organic peroxides. Thus they play a major role in propagating free radical reactions and consequently in determining the degree of free radical pathology. Furthermore, 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+}, act as

effective enzyme inhibitors. Recent studies (Stohs and Bagchi, 1995) have reported that the metals, including iron, copper, chromium and vanadium, undergo redox cycling; while cadmium, mercury, nickel and lead deplete glutathione, protein-bound sulfhydryl groups resulting in the production of reactive oxygen species as superoxide ion, hydrogen peroxide and hydroxyl radicals. As a consequence, enhanced lipid peroxidation, DNA damage and altered calcium and/or sulfhydryl homeostasis occurs. Fenton like reactions may be commonly associated with most membrane fractions including mitochondria, microsomes and peroxisomes. 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 quinones and related compounds may require metal ions. Metal ions may 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).

There are reports suggesting the involvement of lead in initiation and propagation of free radical reactions. The role of lead in induction of lipid peroxidation by producing various reactive oxygen species in various organs is highlighting another mechanism of lead toxicity. Lead and cadmium cause cell death by accelerating iron dependent lipid peroxidation (Quinlan et al., 1988).

It has been reported (Columbano et al., 1983) that intramuscular injection of lead acetate (10 mg/kg, b. wt.) daily for 7 days produces the lipid peroxidation and proliferation of hepatic cell. Marked elevation in hepatic lipid peroxidation and decreased enzymatic antioxidants such as SOD and CAT as well as non-enzymatic antioxidants such as total sulfhydryl group and glutathione has been also reported (Patra et al., 2001).

Cadmium intoxication induces oxidative stress as well and alters the antioxidant system resulting in oxidative damage. Under specific conditions, cadmium can induce a pro-oxidant stage in biological systems resulting in the peroxidation of polyunsaturated fatty acids (Thevenod, 2000).

2.3 ANTIOXIDANTS

To counter oxidative stress, cells constitutively express enzymes that detoxify the reactive oxygen species and repair the damage caused by them. The defenses are

inadequate, however, if the rates of intracellular $O_2^{-\bullet}$ and H_2O_2 formation are accelerated.

Fortunately, the human body makes several important antioxidants. The most important are ubiquinol 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, and vitamin E and trace metals such as selenium.

An antioxidant is any substance that, when present at low concentrations 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.

Antioxidants are compounds capable of providing free radicals with the electrons they are missing while remaining stable themselves, thus preventing a cascade of interactions that could create even more free radicals (Cotgreave et al., 1988). There are two types of antioxidants:

Endogenous antioxidants: Those which are physiological in origin and

Exogenous antioxidants: Those are not be produced by the human body but may protect against pro-oxidant forces when administered as supplement (Sen, 1995).

Superoxide dismutase, catalase, glutathione peroxidase, glutathione-s-transferase are some of the more important endogenous antioxidants.

For the past ten years, several reports touting the miraculous health benefits of vitamin E, C and carotenoids have been published. Vitamin E and vitamin C are essential enzyme cofactors and nourish cells and cellular constituents but are often also regarded as important *in vivo*, protecting against free radicals by scavenging reactive oxygen species. In addition to enzymes, many vitamins are antioxidants in their own right, such as vitamin E, vitamin C, beta-carotene, leutein, lycopene, vitamin B_3 in the form of niacin, vitamin B_{12}, vitamin B_6, co-enzyme Q10 and cysteine (amino acid), many herbs, such as bilberry, turmeric, grape seed extract and pine bark extract and ginkgo (Ames et al., 1993). Some microbial products like *spirulina* and its species can also provide powerful antioxidant protection for the

body. A wide variety of antioxidant vitamins, minerals and herbs may be the best way to provide the body with the most complete protection against free radicals damage. *Spirulina,* as an recently known antioxidant, being natural algae very rich in proteins and other essential material, need to be focused as an antioxidant. Reports showing antioxidant property of *spirulina* is the first step in this current context (Shastri et al., 1999). *Spirulina* is rich in antioxidants including provitamin A (beta-carotene) and SOD enzyme (Henrickson, 1989), the presence of these two considered making an effective system for the prevention of harmful effects by physical and chemical agents (Shastri et al., 1999).

2.4 EXOGENOUS ANTIOXIDANTS IN METAL INDUCED OXIDATIVE STRESS

α-tocopherol is a lipid soluble molecule, which functions as the most important lipid peroxidation chain breaking stabilizer. α-tocopherol is believed to act as an antioxidant, protecting membrane-bound polyunsaturated fatty acids (PUFA) and other oxygen sensitive substances, such as vitamin A and vitamin C, from oxidation by reacting with free radicals before they bind with cell membrane lipids. Ascorbate is an essential enzyme cofactor but is often regarded as an important antioxidant *in vivo*, protecting against cancer by scavenging DNA damaging ROS. Vitamin C decreases the deposition of lead and cadmium in liver, kidney and bone (femur) (Dalley et al., 1989) and decreases the retention and toxicity of lead and cadmium (Flanagan et al. 1982).

Spirulina, a member of the family Oscillatoriaceae, is multi-cellular, filamentous and non-heterocytous blue green algae. It has been reported that alcohol and water extracts of *spirulina* effectively inhibit lipid peroxidation. *Spirulina* was reported to reduce the lead induced toxicity that is attributed to the presence of the antioxidants, β-carotene and SOD enzyme (Shastri et al., 1999).

2.5 AIMS AND OBJECTIVES

Recent evidences (Ding et al., 2000; Manca et al., 1991; Rikans and Yamano, 2000; Fariss, 1991) have shown that lead and cadmium induces oxidative stress by

generating ROS, reducing the antioxidant defense system of cells via depleting glutathione, inhibiting sulfhydryl dependent enzymes, interfering with some essential metals needed for antioxidant enzyme activities, and/or increasing susceptibility of the cells to oxidative attack by altering the membrane integrity and fatty acid composition. Consequently, it is plausible that impaired oxidant/ antioxidant balance can be partially responsible for the toxic effect of lead and cadmium: where enhanced oxidative stress contributes to lead and cadmium induced toxicity. Restoration of a cells' antioxidant capacity appears to provide a partial remedy. Several studies are underway to determine the effect of antioxidant supplementation following lead and cadmium exposure.

Overall high reactivity of endogenous and exogenous antioxidants with radicals may be beneficial *in vivo* e.g. by scavenging peroxyl radicals and inhibiting lipid peroxidation. If reactive oxygen species are intimately involved with the redox regulation of cell functions, as seems likely from current evidence, it may be easier to understand why attempts to change antioxidant balance in various experiments have been made, there must be a highly complex interrelationship between dietary constitutive, and inducible antioxidant with the body, under genetic control. The challenge for the new century is to be able to understand these relationships, and how to manipulate them to owing the advantage to prevent and treat diseases or disorders. Recently, it has been reported (Halliwell, 1999) that diets rich in fruits and vegetables are associated with decreased risk of free radicals. Biomarkers of oxidative DNA damage and lipid peroxidation can also be used to establish the role of antioxidants in this protection and the optimal intake of these constituents.

The exhaustive literature review and reports (Halliwell and Gutteridge, 1986) by researchers and most medical practitioners have now indicated the free radicals as the culprit behind most of the disorders and diseases. The generation and control of these free radicals depend upon the oxidative stress, enzymatic and non-enzymatic body defenses, metal ions and antioxidants. In normal circumstances, free radicals are controlled and quenched by endogenous antioxidants like superoxide dismutase, catalase, reduced glutathione etc.

However, the body's own mechanisms to combat the onslaught of free radicals becomes inadequate when the rates of free radical generation are highly accelerated for e.g. by catalytic effects of transition metals ions. Of these metals, lead and cadmium are very important due to their ubiquitous occurrence in air,

water, soil and food etc. Studies have implicated their role not only in lipid peroxidation but also in reduction of endogenous antioxidant levels.

There is a need for supplementation of exogenous antioxidants to protect the body from the onslaught of free radical induced damage, in such conditions.

Vitamin E, vitamin C and *spirulina* have emerged as the proven and effective antioxidants for combating free radicals induced stress to the body. Many *in vitro* studies have reported the release of free radicals and decrease in endogenous antioxidants by lead and cadmium (Eickhoff et al., 1995; Ding et al., 2000; Schluter et al., 1995), which cannot be directly correlated with *in vivo*.

Hence our aim was to study the effect of lead and cadmium induced oxidative stress on endogenous enzymes in various organs and systems of the body and to investigate the beneficial effects of exogenous antioxidants like vitamin E, vitamin C and *spirulina* on such a stressful condition.

Therefore the present work **"Biochemical, Pharmacological and Toxicological Investigations of Some Metals with Reference to Oxidative Stress"** is aimed:

- To study the effects of lead and cadmium alone on the levels of various enzymes and cellular constituents in organs like liver, lung, heart and kidney and in serum of rats with reference to oxidative stress.
- To find out the scope for exogenous antioxidants like vitamin E, vitamin C or *spirulina* supplementation on the lead and cadmium induced changes in the levels of various enzymes and cellular constituents in tissues and serum with reference to oxidative stress.
- To study the toxicological effects of these metals alone and in combination with vitamin E, vitamin C or *spirulina* on the basis of biochemical and pharmacological evidence.

The investigations carried out are:

1. **Tissues**

   A. Lipid peroxidation

   Malondialdehyde, Hydroperoxide and Conjugated dienes in liver, kidney, lung, heart and brain.

   B. Endogenous antioxidant, other enzymes and cellular constituents

   Superoxide dismutase, Catalase, Reduced glutathione in liver, kidney, lung, heart and brain.

   Glucose-6-phosphate dehydrogenase in liver.

   Sodium potassium ATPase, Magnesium ATPase, Calcium ATPase in liver and kidney.

   Inorganic phosphorous in liver, kidney, lung and heart.

   Total Protein in liver, kidney, lung, heart and brain.

   C. Lipids

   Cholesterol, Triglycerides and Phospholipids in liver, kidney, lung and heart.

   D. Metals

   Lead and Cadmium in liver, kidney, lung, heart and brain.

2. **Serum**

   Glutamate pyruvate transaminases, glutamate oxaloacetate transaminases, alkaline and acid phosphatases, lactate dehydrogenase, total bilirubin, proteins and serum lipids (cholesterol, triglycerides and phospholipids).

Apart from this, body weight, organ weight and histopathological studies were also carried out.