INTRODUCTION

Oxidative Stress represents a biochemical state of excessive presence of free radicals and reactive metabolites that exhibit harmful biological effects potentially damaging the organism. Free radicals are molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular Orbitals that confer the molecules considerable reactivity ***(Durackova, 2010).***

Reactive oxygen species (ROS) (for example, superoxide anion radical($O_2^{\cdot -}$), hydroxyl radical($^{\cdot}OH$) and hydrogen peroxide (H_2O_2),..etc) are products of normal cellular metabolisms. At low or moderate concentrations, they actually play an important beneficial role in certain physiological processes, such as cellular responses in defense against infectious agents and certain normal cellular signaling activities. Their excessive accumulation can cause damage of biological molecules and systems.

The normal and important balance between beneficial and harmful effects of Reactive oxygen species (ROS) in the body is maintained by redox regulation mechanisms that protect the body from oxidative stress (OS). Thus, oxidative stress (OS) represents an imbalance between the production of oxidants (ROS) and their elimination by the protective antioxidative systems in the body ***(Droge, 2002).***

Glutathione-*S*-transferase (GSTs) is one of the important constituents of Cellular antioxidant enzyme system, belongs to the super family of enzymes that plays vital role in decomposition of lipid hydro-peroxides formed due to ROS induced peroxidation of the membrane lipids. It also catalyzes the conjugation of reduced glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds, the first step in mercapturic acid pathway that leads to the elimination of toxic compounds ***(Hayes et al., 2005).***

The determination of erythrocyte GSTactivity is a promising indicator of oxidative stress conditions that occur in various types of anemia ***(Chiang et al., 2007).***

Iron is an essential micronutrient, as it is required for an adequate erythropoietic function, oxidative metabolism and cellular immune response ***(Munoz et al., 2010).***

Iron exists in two forms ferrous and ferric state, the ferrous ($Fe^{2+)}$ state, is the biologically active form of the element. The redox active, Ferrous(Fe2+), catalyses the generation of a powerful free radical – the hydroxyl radical – by the Fenton reaction ***(Galaris et al., 2008),*** resulting in an increase in oxidative stress and damage to cellular macromolecules. Hence, in plasma there is normally limited bioavailability of free iron due to iron sequestration in transport and storage proteins ***(Campenhout et al., 2006).***

Iron deficiency is the leading nutritional deficiency in the world and the most common cause of anemia. It is more common in developing countries, affecting from 30% to 70% of the population where malnutrition and infections are prevalent ***(Andrews, 2009).***

In iron deficiency anaemia, although the levels of free iron in the circulation are found to be normal or low, there is enhanced oxidative stress ***(Yoo et al., 2009).*** The deficiency of iron causes tissue hypoxia and also affects the production of iron-containing antioxidant proteins which tilts the balance to the oxidative side ***(Toxqui et al., 2010).***

Iron deficiency also affects mitochondrial oxidative phosphorylation leading to decreased ATP production and causes loss of structural and functional integrity of the cell ***(Parasuram et al., 2010).*** In addition, impairment of the antioxidant defense system and decreased cellular immunity have also been reported in patients with iron deficiency anemia ***(Toxqui et al., 2010).*** So Alterations in the pro oxidant/antioxidant balance are considered to be the cause of oxidative stress ***(Yoo et al., 2009).***

The correction of iron deficiency with oral iron supplementation leads to rejuvenation of defective antioxidant system and brings back the balance between Reactive oxygen species (ROS) and antioxidant system ***(Parasuram et al., 2010).***

Antioxidants are protective agents that inactivate Reactive oxygen species (ROS) and play an essential role in protection of the cells from oxidative damage. They include enzymatic agents (glutathione peroxidase, superoxide dismutase, catalase), and non enzymatic agents (uric acid, glutathione, bilirubin, ascorbic acid,vitamin A, and vitamin E) ***(Dhawan et al.,2005).***

A debate is still held about adding synthetic antioxidants in the treatment of children with IDA that results in decrease of lipid peroxidation, prevention of pathologic progression and rapid improvement of clinical manifestations ***(Shved et al., 1995, Parasuram et al., 2010).***

Aim of the Work

To determine the oxidative stress state in infants with iron deficiency anemia and the effect of iron therapy with or without antioxidant treatment in the reversal of this state.

Chapter One

OXIDANT AND ANTIOXIDANT (REDOX) HOMEOSTASIS

Introduction about normal balance and Redox homeostasis

Redox (reduction and oxidation) reactions are a family of reactions that are concerned with the transfer of electrons between molecules. Normally, the redox reactions ensure that the cells respond properly to endogenous and exogenous stimuli. During the cellular redox process, ***Reactive Species*** - Oxygen (ROS) and nitrogen (RNS) - are liberated as byproducts ***(Sherki et al., 2001),*** Under physiologic conditions, cells maintain redox balance through generation and elimination of reactive oxygen/nitrogen species (ROS/RNS) ***(Halliwell, 2006).***

Reactive oxidizing molecules are the molecules with a strong oxidizing property. ***Free radicals***, also called as radicals, are molecules which have unpaired electrons in their outer orbits. Because of this property, they are highly reactive. There are other molecules which lack free electrons in their outer orbits but are highly reactive. These molecules are called as non free radicals. Because of their high reactivities, the free and the non free radicals are collectively called as ***Reactive Species (Valko et al., 2007; Durackova, 2010).***

Though many reactive molecules are formed in the body, the Reactive Oxygen Species (ROS) and the Reactive Nitrogen Species (RNS) are the important ones.

ROS and RNS are formed under normal physiological conditions as the products of the cellular metabolism ***(Valko et al., 2007).*** They are well recognised for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems ***(Valko et al., 2006).***

Reactive oxygen species (ROS) produced at low or moderate concentrations actually play an important beneficial role in certain physiological processes, such as cellular responses in defense against infectious agents, certain normal cellular signaling activities and in the induction of a mitogenic response. Excessive accumulation of ROS can cause damage of biological molecules and systems ***(Dröge, 2002; Ridnour et al., 2005).***

The delicate balance between beneficial and harmful effects of free radicals is a very important aspect of living organisms and is achieved by mechanisms called "redox regulation". The process of "**redox regulation**" protects living organisms from various oxidative stresses and maintains "**redox homeostasis**" by controlling the redox status in vivo ***(Dröge, 2002).***

Mechanisms of maintenance of reduction/ oxidation "redox homeostasis":

Free radicals and reactive diamagnetic species derived from radicals operate at low, but measurable concentrations in the cells. Their "steady state" concentrations are determined by the balance between their rates of production and their rates of removal.

The redox state of a cell is kept within a narrow range under normal conditions, similar to the manner in which a biological system regulates its pH ***(Schafer & Buettner, 2001).***

The intracellular "redox homeostasis" or "redox buffering" capacity is substantiated primarily by glutathione GSH and thioredoxin (TRX). The glutathione (2GSH/GSSG couple) represents the major cellular redox buffer and therefore is a representative indicator for the redox environment of the cell ***(Schafer & Buettner, 2001; Dröge, 2002).***

Under enhanced oxidative stress conditions, GSSG content increases, this in turn increases the content of protein mixed disulphides. The high ratios of reduced to oxidised GSH and TRX are maintained by the activity of GSH reductase and TRX reductase, respectively and both of these "redox buffering" thiol systems counteract intracellular oxidative stress ***(Dröge, 2002).***

In addition to antioxidant functioning in the cell, GSH and TRX are involved in cell signalling process ***(Dröge, 2002).***

Thioredoxin (TRX) inhibits apoptosis signaling by scavenging intracellular ROS in cooperation with the GSH system. Mitochondria-specific thioredoxin (TRX-2) and TRX peroxidases (peroxiredoxins) are suggested to regulate cytochrome release from mitochondria, which is a critical early step in the apoptosis-signaling pathway ***(Masutani et al., 2005)***. As mitochondria are the most redox-active organelles and indispensable for cells to initiate or inhibit the apoptosis process, the regulation of mitochondrial function is the central focus in the research field of apoptosis and redox homeostasis ***(Ueda et al., 2002).***

A) Oxidative Stress

Oxidative stress can be defined as an imbalance between pro-oxidants and antioxidants leading to free radical formation ***(Tandon and Garg, 2011).*** It represents a biochemical state of excessive presence of free radicals and reactive metabolites that exhibit harmful biological effects potentially damaging the organism ***(Valko et al., 2007)***.

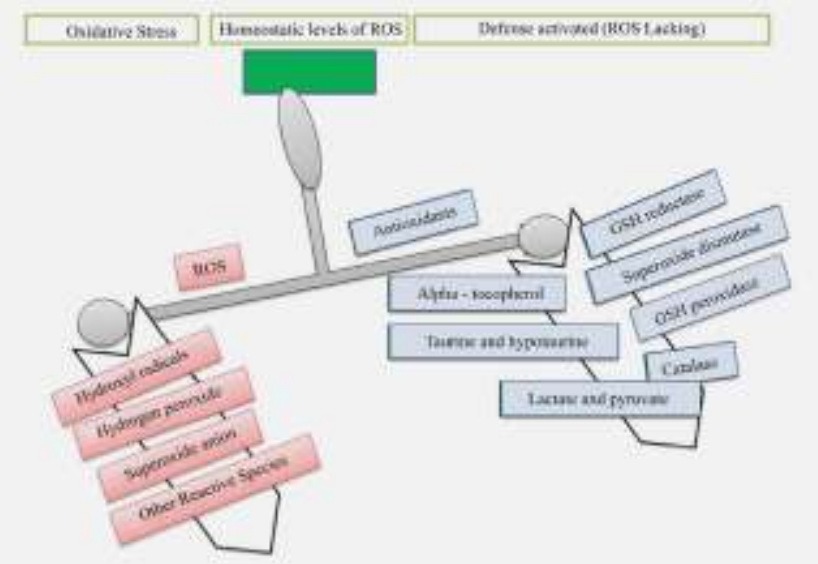

Figure (1): Imbalance between oxidant and antioxidant ***(Rahman et al., 2012).***

Sources of free radicals:

I- Endogenous sources:

- ***Normal aerobic metabolism in mitochondria:***

The source of most, if not all, cellular ROS in a normal oxidation state (not stressed) is mitochondrial respiratory chain ***(Modlinger et al., 2004).***

Approximately 90% of the oxygen utilized by the cell is consumed by the mitochondrial electron transport system ***(Sharma et al., 2010).***

Electron transport chain produces superoxide anion in mitochondria by the reduction of molecular oxygen. Molecular oxygen is a powerful oxidizing agent and, as such, is an

excellent terminal acceptor of electrons in the electron transport chain. "In the textbook scheme of mitochondrial respiration, electron transport involves a coordinated four-electron reduction of O2 to H2O". However, mitochondrial electron transport is imperfect and, thus, a small percentage of electrons engaged in the electron transport chain react with diatomic oxygen, resulting in the superoxide anion. The superoxide anion can, in turn, be converted into the much less reactive hydrogen peroxide through reaction catalyzed by superoxide dismutase (SOD). However, when hydrogen peroxide interacts with ions of transition metals such as iron and copper, the most reactive ROS, hydroxyl radicals ($OH^{\bullet}$) are formed **(Fenton's reaction)** ***(Kowaltowski et al., 2001; Fukui and Moraes, 2008).***

- ***Phagocytic cells:***

If a phagocytic cell such as the neutrophil is exposed to a stimulus, it has the ability of recognising the foreign particle and undergoing a series of reactions called the respiratory burst (Oxidative burst) ***(DeCoursey & Ligeti, 2005).***

Macrophages and neutrophils contain a group of enzymes called the Nicotine adenine dinucleotide phosphate (NADPH) oxidase complex ***(De la Rosa, 2006).*** Phagocyte NADPH oxidase *(best characterised in neutrophils*) plays an important role in host defenses against invading microbes by producing superoxides generating the respiratory burst necessary for

microbial destruction ***(Ghafourifar&Cadenas, 2005; Prata et al., 2006).***

Activated macrophages initiate an increase in oxygen uptake that gives rise to variety of ROS, including $O_2^{\bullet-}$, $NO^{\bullet}$ and H_2O_2 ***(De la Rosa, 2006).***

- ***Myeloperoxidase:***

This is heme-containing enzyme, present in neutrophils and eosinophils and catalyzes the H2O2 with various substrates to form highly reactive hypochloric acids ***(Klebanoff, 2005).***

- ***Cytochrome p450 enzyme:***

It metabolizes a variety of substrates such as fatty acids, cholesterol and drugs (e.g. acetaminophen). The underlying biochemical reactions consume oxygen whereby small amounts of ROS are generated ***(De la Rosa, 2006).***

- ***Transitional metals (The Fenton Reaction):***

The redox state of the cell is largely linked to an iron (and copper) redox couple and is maintained within strict physiological limits. It has been suggested that iron regulation ensures that there is no free intracellular iron; however, in vivo, under stress conditions, an excess of $O_2^{\bullet-}$ releases "free iron" from iron-containing molecules.

$$\mathbf{Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2} \quad \text{eq. (I)}$$

The released Fe^{2+} can participate in the Fenton reaction, generating highly reactive $OH^{\bullet}$:

$$\mathbf{Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}}$$ eq. (II)(Fenton reaction)

(Valko et al., 2005)

$O_2^{\bullet-}$ participates in the Haber–Weiss reaction which combines a Fenton reaction and the reduction of Fe^{3+} by $O_2^{\bullet-}$ yielding Fe^{2+} and oxygen.

$$\mathbf{O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^{-}}$$ eq.(III) (Haber–Weiss reaction).

However, this reaction depends on the presence of transition metals such as Cu^{+} and/or Fe^{2+}, which work as reducing agents ***(Liochev and Fridovich, 2002).***

- ***Nitric Oxide (NO):***

Nitric oxide is generated in biological tissues by specific nitric oxide synthases (NOSs), which metabolise arginine to citrulline with the formation of NO• via a five-electron oxidative reaction ***(Ghafourifar & Cadenas, 2005).*** Nitric oxide (NO•) is an abundant reactive radical that acts as an important oxidative biological signaling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation and immune regulation ***(Bergendi et al., 1999).***

Cells of the immune system produce both the superoxide anion and nitric oxide during the oxidative burst triggered during inflammatory processes. Under these conditions, nitric oxide and the superoxide anion may react together to produce significant amounts of a much more oxidatively active molecule, peroxynitrite anion (ONOO−), which is an oxidizing free radical that can cause DNA fragmentation and lipid oxidation ***(Carr et al., 2000):***

$NO\bullet + O_2\bullet^- \longrightarrow ONOO^-$ (1)

- ***Peroxisomes:***

Peroxisomes are major sites of oxygen consumption in the cell and participate in several metabolic functions that use oxygen. Oxygen consumption in the peroxisome leads to H_2O_2 production, which is then used to oxidize a variety of molecules. The organelle also contains catalase, which decomposes hydrogen peroxide and presumably prevents accumulation of this toxic compound. Thus, the peroxisome maintains a delicate balance with respect to the relative concentrations or activities of these enzymes to ensure no net production of ROS. When peroxisomes are damaged and their H_2O_2 consuming enzymes are downregulated, H_2O_2 is released into the cytosol which is significantly contributing to oxidative stress ***(DeCoursey & Ligeti, 2005).***

Peroxisomal oxidation of fatty acids has recently been recognised as a potentially important source of H_2O_2 production during prolonged starvation ***(De la Rosa, 2006).***

- ***Xanthine oxidase (XO):***

XO is an iron containing hydroxylating enzyme involved in the degradation of purine like nucleotides. This enzyme catalyzes the reaction in which hypoxanthine is converted to xanthine and xanthine to uric acid. In both steps, molecular oxygen is reduced, forming the superoxide anion in the first and hydrogen peroxide in the second ***(Valko et al., 2004).***

- ***Heme proteins:***

Oxidation of oxyhemoglobin and oxymyoglobin to their met forms is accompanied by release of heme-bound superoxide ***(Halliwell, 2006)***.

II- Exogenous sources:

They include exposure to environmental or pathological agents such as atmospheric pollutants, cigarette smoking, ultraviolet rays, radiation, toxic chemicals, viral & bacterial infections ***(Videla, 2009).***

Environmental stresses can cause metabolic changes in humans by either increasing the production of reactive oxygen and nitrogen species (RONS) or decreasing the antioxidant production ***(Alessio and Hangerman, 2006).***