CHAPTER I
3.1.0.0. INTRODUCTION

Molecular oxygen is an essential nutrient for higher forms of life. In addition to its normal physiological reactions, oxygen and its partially reduced forms, ROS oxidize a variety of macromolecular and simpler compounds in cells and fluids of the body. When ROS production is excessive, such as during prolonged aerobic exercise, or when antioxidant defense is severely hampered by nutritional deficiencies, or pharmacological intervention, an inadequate defense may be overwhelmed by the ROS, leading to extensive cell and tissue damage (Esterbauer et al., 1991).

When PUFAs on the biomembranes are attacked by free radicals in the presence of molecular oxygen, a chain of peroxidative reactions occur, eventually leading to the formation of hydrocarbon gases and aldehydes (eg: malondialdehyde). Byproducts of lipid peroxidation are the most frequently studied markers of oxidative tissue damage. As a protection against excessive oxidation, nature has developed a complex set of interactive antioxidant systems. Biological antioxidants include SOD, catalase, γ-glutamyl cycle enzymes like Se-GSH Px and glutathione reductase. Nonenzymatic biological antioxidants include tocopherols, ascorbic acid and glutathione. Nutritional deficiency provides an excellent model to study the dynamic balance between oxidative challenge and antioxidant defense in the biological system. Among the nutritional deficiencies, vit.E and Se form the most important dietary factors contributing to tissue antioxidant defenses.

Vitamin E functions as an important cellular antioxidant in the hydrophobic compartments, protecting PUFAs from lipid peroxidation (Burton and Ingold, 1989; Kagan et al., 1989). Consequently dietary vit.E deprivation and/or exercise may result in increased lipid peroxidation products such as MDA and possible disruption of membrane function (Sjodin et al., 1990; Tiidus et al., 1993). Selenium has been recognized as an essential trace element in animal nutrition playing a protective role against peroxidative damage in the animal cells, mainly in
the form of GSH Px (Sunde and Hockstra, 1980). Se deficiency is known to result in the loss of GSH Px activity, the enzymes involved in the reduction of hydroperoxides (Hill et al., 1987; Ji et al., 1988). The role of vit.E and Se in the prevention of coronary heart disease (Van-Poppel et al., 1994; Luoma et al., 1995), muscle damage and soreness (Kanter, 1994), cystic fibrosis (Kauf et al., 1995) and cancer (Schwartz et al., 1994) has been extensively studied. Although liver is equipped with abundant antioxidant enzymes and other scavenging systems, the high metabolic rate and central role in detoxification make it one of the main targets for free radical damage in the body, more so under impaired antioxidant defenses. Animals fed on diet deficient in vit.E and/or Se nutrients develop a number of pathological conditions (Scott, 1978). In addition to respiratory function, the lung offers a first line of defense against environmental toxicants, pollutants and oxidants. Lung is the principal target organ for oxygen poisoning in mammals. Severe morphological and functional alterations in the lung are caused by hyperoxic conditions, often resulting in death (Crapo et al., 1980b). Selenium deficiency has been shown to augment the pulmonary toxic effects of oxygen exposure in the rat (Hawker et al., 1993).

A stable intracellular redox state is of vital importance to organisms (Krentzchmar and Klinger, 1990). Many enzymes require the essential thiols to be kept in reduced state. As the most important non-protein thiol source, GSH concentration in the cell is remarkably high. Liver is the major organ for de novo GSH synthesis and supplies 90% of the circulating GSH (Potter and Tran, 1993). From the studies in mice and rats, liver has been recognized as the central organ of the interorgan homeostasis of GSH, serving as the principal source of GSH for plasma (Lauterburg et al., 1984). Lung, like liver and kidney shows a high rate of GSH turnover (Martensson et al., 1989). Studies in isolated perfused rat lung indicate use of plasma GSH by the lung. Thus GSH and associated enzyme systems play an important role in mediating cellular antioxidant defenses. Liver and lung are the main tissues vulnerable to oxidative damage in animals subjected to oxidative stress. Hence in the present study the rate of lipid peroxidation and
the metabolism of glutathione in liver and lung tissues were analyzed in animals fed on vit.E and Se deficient diets in order to assess the oxidative stress induced in relation to the animals fed on vit.E and Se supplemented diets.

3.2.0.0. RESULTS

Wistar strain female albino rats at weanling stage were divided into four groups of 12 animals each and they were fed on -E+Se, +E-Se, -E-Se and +E+Se diets for a period of 13 weeks. After the dietary treatment period, animals were killed by cervical dislocation. Liver and lung tissues were excised after perfusion with physiological saline and kept in -80°C until used for biochemical analysis.

3.2.1.0. Vitamin E

Feeding vit.E deficient (-E+Se, -E-Se) diet for a period of 13 weeks to wistar strain female albino rats, starting from the weanling stage, resulted in a significant decrease in vit.E levels both in liver and lung tissues when compared to supplemented animals (+E-Se and +E+Se) (Fig. 14A&B). Vitamin E levels in these tissues fell to approximately 10% of levels of +E+Se animals.

3.2.1.2. Selenium

Similarly feeding animals with Se deficient diets (+E-Se and -E-Se) resulted in significant depletion of Se both in lung and liver tissues (Fig. 15A&B). Selenium levels in the tissues of Se deficient animals fell to 5% of levels in Se supplemented animals.

3.2.1.3. Growth and tissue somatic indices

The dietary treatment given for a period of 13 weeks starting from weanling stage resulted in significant changes in the growth pattern (Fig. 16) and tissue somatic indices (Fig. 17A&B). Animals fed on vit.E and Se supplemented diets (+E+Se) maintained the highest growth pattern compared to all other groups,
Fig. 14: Vitamin E levels in liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets for 13 weeks.

Each value is the mean + SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another (p < 0.05).
Fig. 15: Selenium levels in liver and lung tissues of rats fed on vit. E and/or Se supplemented and deficient diets

Each value is the mean + SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another (p < 0.05).
Fig. 16: Growth curves of rats fed on vit.E and/or Se supplemented and deficient diets

Each value is the mean + SD of 12 individual observations.
Fig. 17: Tissue somatic indices of rats fed on vit.E and/or Se supplemented and deficient diets

Each value is the mean + SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another (p < 0.05).
throughout the period of dietary treatment. Animals fed on -E-Se diets, on the other hand, showed the reduced growth rate compared to all the other groups studied. The other two groups (+E-Se and -E+Se) showed intermediate growth rate between +E+Se and -E-Se groups. Tissue somatic indices (TSI) of both liver and lung tissues were significantly lower in the +E+Se group of rats compared to all other groups (Fig. 17A&B).

3.2.1.4. Lipid Peroxidation

RP-HPLC analysis was used to separate lipid peroxides in the form of MDA adduct from other chromogens absorbing at 532 nm. Use of HPLC eliminates artifacts due to the reaction of TBA with other body-fluid constituents to give different chromogens. As shown in Fig. 18A, significantly higher concentrations of MDA were observed in liver tissue of -E+Se (32%), +E-Se (106%) and -E-Se (136%) group of animals when compared to supplemented (+E+Se) animals. Similarly in lung tissue also higher levels of MDA were observed in -E+Se (35%), +E-Se (161%) and -E-Se (200%) animals when compared to supplemented animals (Fig. 18B).

3.2.2.0. Antioxidant defense systems

The levels of cytosolic antioxidant enzymes like Se-glutathione peroxidase, \( \gamma \)-glutamylcysteine synthetase, glutathione reductase, \( \gamma \)-glutamyl transpeptidase and glutathione S-transferases were measured in liver and lung tissues of rats fed on the different diets. The levels of GSH and GSSG were determined in perfused tissues immediately after isolation.

3.2.2.1. Se-glutathione peroxidase

Se-GSH \( \Gamma x \), the enzyme involved in the reduction of inorganic and organic hydroperoxides, activity levels reached to insignificant levels in liver and lung tissues of Se deficient animals (+E-Se and -E-Se) compared to the Se supplemented ones (-E+Se and +E+Se) (Fig. 19A&B). This observation suggests
Fig. 18: Lipid Peroxidation in liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets

Each value is the mean + SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another (p < 0.05).
Fig. 19: Activity levels of Se-GSH Px with $\text{H}_2\text{O}_2$ in the cytosolic fractions of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets.

Each value is the mean ± SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another (p < 0.05).
the efficiency of dietary treatment in depleting tissue Se levels and thereby decreasing the activity levels of Se-dependent enzymes.

### 3.2.2.2. Reduced glutathione

The levels of both GSH and GSSG were determined simultaneously on RP-HPLC. In liver tissue GSH levels were significantly higher in +E-Se (66 %) and -E-Se (83.4%) animals when compared to +E+Se animals. In contrast, GSH levels in lung tissue were significantly lower in -E+Se (-24%), +E-Se (-41.8%) and -E-Se (-55.22%) animals when compared to +E+Se animals (Fig. 20A&B).

#### 3.2.2.3. Oxidized glutathione

In liver and lung tissues the levels of GSH were 5 fold higher than GSSG levels. GSSG levels in liver of Se deficient animals were significantly lower (-45% in +E-Se and -45.7% in -E-Se) when compared to Se supplemented (-E+Se and +E+Se) animals (Fig. 21 A). However in lung tissue GSSG levels were significantly higher in -E+Se (70%), +E-Se (181%) and -E-Se (216%) animals when compared to that of +E+Se animals (Fig. 21B).

### 3.2.2.4. γ-Glutamylcysteine synthetase

Glutamylcysteine synthetase activity levels in both liver and lung tissues of -E+Se, +E-Se, and -E-Se animals were significantly higher compared to +E+Se groups (Fig. 22A&B). In liver tissue GCS levels were significantly higher in -E+Se (70%), +E-Se (109%) and -E-Se (153%) group of animals. Similarly, in lung tissue higher levels of GCS were observed in -E+Se (40%), +E-Se (197%) and -E-Se (210%) groups when compared to +E+Se animals.

### 3.2.2.5. Glutathione reductase

Glutathione reductase activities in the present study were increased in the liver and lung of all deficient rats when compared to supplemented animals. The
Fig. 20: Levels of GSH in liver and lung tissues of rats fed on vit.F. and/or Se supplemented and deficient diets

Each value is the mean + SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another (p < 0.05).
Fig. 21: Levels of GSSG in liver and lung tissues of rats fed on vit £ and/or Se supplemented and deficient diets

Each value is the mean + SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another (p < 0.05).
Fig. 22: Activity levels of γ-glutamylcysteine synthetase in the cytosolic fractions of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets

Each value is the mean + SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another (p < 0.05).
hepatic glutathione reductase activities were significantly higher in -E+Se (46%), +E-Se (61%) and -E-Se (81%) animals when compared to that of +E+Se animals (Fig. 23A). Similarly in lung tissue, all deficient groups showed significantly higher levels (40% in -E+Se, 63% in +E-Se and 81% in -E-Se) compared to +E+Se animals (Fig. 23B).

3.2.2.6. \(\gamma\)-Glutamyl transpeptidase

Liver, in spite of the highest concentration of GSH showed lesser GGT activity compared to lung tissue. GGT activity levels were significantly reduced in liver tissue of vit.E and/or Se deficient animals when compared to +E+Se animals (Fig. 24A). In contrast, GGT activity levels in the lung tissue of vit.E and/or Se deficient animals were significantly higher compared to that of +E+Se animals (Fig. 24B).

3.2.2.7. Glutathione S-transferases

The hepatic GST activities were significantly higher in +E-Se (48.5%) and -E-Se (77%) animals when compared to that of +E+Se animals (Fig. 25A). However in lung tissue all deficient groups showed significantly higher levels (48% in -E+Se, 40% in +E-Se and 80% in -E-Se) compared to +E+Se animals (Fig. 25B).

3.3.0.0. Discussion

Vit.E, as a chain breaking free radical scavenger, protects the tissue from non-enzymatic lipid peroxidation. Vh.E has been shown to be involved in the inhibition of LOX (Reddanna et al., 1989, Lomnitski et al., 1991) and in the reduction of hydroperoxides catalyzed by LOX (Cucurou et al., 1991). Thus vit.E plays an important antioxidant role both against enzymatic and non-enzymatic lipid peroxidations. Selenium, as an integral component of Se-GSH Px, plays an important role in the reduction of organic and inorganic hydroperoxides.
Fig. 23: Activity levels of glutathione reductase in the cytosolic fractions of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets

Each value is the mean + SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another (p < 0.05).
Fig. 24: Activity levels of γ-glutamyl transpeptidase in the cytosolic fractions of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets

Each value is the mean + SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another (p < 0.05).
Fig. 25: Activity levels of GSTs with CDNB in the cytosolic fractions of liver and lung tissues of rats fed on vit.E and/or Se supplemented and deficient diets

Each value is the mean ± SD of six individual observations. Data was analyzed with a one way ANOVA followed by SNK test. Bars labelled with same letter are not significantly different from one another (p < 0.05).
In view of their key role in tissue antioxidant defenses, vit.E and/or Se deficiency markedly reduced the growth rate of female albino rats (Fig. 16). As a result of this liver and lung tissue somatic indices also got affected (Fig. 17). The increase in the TSI of liver and lung tissues in vit.E and/or Se deficient animals in comparison to the supplemented group, is more due to the decrease in the body weights rather than increase in tissue weights.

The impaired antioxidant defenses as a result of induced vit.E and Se deficiency could be responsible for the observed increase in lipid peroxides in animals deficient in Se and/or vit.E (Fig. 18A). In vivo, MDA is formed almost exclusively from peroxidation of PUFAs, hence its presence is thought to be a prime indicator of the occurrence of tissue lipid peroxidation (Gutteridge and Halliwell, 1990). Similar increase in lipid peroxides was reported in liver, lung and cardiac muscles of animals fed on vit.E and/or Se deficient diets (Hafeman and Hoekstra, 1977; Tiidus et al., 1993; Awad et al., 1994; Rokizki et al., 1994). The present study also has clearly demonstrated that liver and lung tissues of vit.E and/or Se deficient animals are vulnerable to impaired antioxidant defenses. Se-GSH Px and other Seleno peroxidases play a prominent role in the reduction of both inorganic and organic hydroperoxides in different compartments of cells. Selenium deficiency in the present study has resulted in significant depletion of Se-GSH Px activity levels (Fig. 19A). Similarly other Seleno peroxidases such as plasma GSH Px and phospholipid hydroperoxidase might have been reduced in Se deficient animals. In addition deficiency of vit.E also might be responsible for the observed increase in the levels of lipid peroxides in both liver and lung tissues of vit.E and/or Se deficient animals (Fig. 18A&B).

In the present study Se deficiency resulted in increased liver GSH levels (Fig. 20A). No significant differences, however, were observed in liver tissue of vit.E deficient animals (Fig. 20A). Hill and Burk (1982) have studied GSH metabolism in isolated rat hepatocytes and demonstrated that GSH synthesis and turnover are accelerated significantly by Se deficiency and not significantly by vit.E
deficiency. This implies that Se deficiency but not of vit.E accelerates GSH synthesis in rat liver. These changes were associated with increased activities of rate-limiting enzymes in the GSH turnover such as GCS in vit.E and/or Se deficient animals (Fig. 22A). Also glutathione reductase, another enzyme involved in GSH turnover, was also at higher levels in liver of vit.E and Se deficient animals (Fig. 23A). This increased glutathione reductase activity might be a mechanism for increasing GSH status to protect liver from the prevailing oxidant stress in nutrient deficient animals. The increased levels of GSH in Se deficient animals could be due to increased demand for GSH caused by increased tissue lipid peroxides.

GGT is an important component of the \textit{y}-glutamyl cycle in cells (Meister and Anderson, 1983). The \textit{y}-glutamyl bond can be cleaved by GGT, an enzyme located on the external surface of cell membranes of various tissues. Liver is known to have negligible GGT activity (Bray and Taylor, 1993). In the present study also GGT activity in liver was much lower compared to lung tissue (Fig. 24A). GGT activity levels in liver were further decreased in vit.E and Se deficient livers when compared to that of +E+Se animals. Hepatic uptake of plasma GSH is very low as a result of the relatively low levels of GGT activity in the liver (Lauterburg et al., 1984). GSH as well as other \textit{y}-glutamyl-containing compounds, including GSSG and \textit{y}-glutamyl glutathione, react with GGT at the outer cell surface. The \textit{y}-glutamyl moiety is transferred to a suitable amino acid acceptor, and both the \textit{y}-glutamyl amino acid and cysteinylglycine are transported into the cell and reused for GCS and glutathione reductase reactions to enhance cellular GSH levels. Although the first step in GSH degradation is catalyzed by GGT, it seems unlikely that hepatic GGT is involved in the reaction in the liver, because of its low level and its further decrease by vit.E and Se depletions. Therefore, GSH release from liver is of primary importance in the GSH turnover. Perhaps, GSH and GSSG released into the blood are cleaved and reabsorbed in kidney and the lung tissues, containing very high activity levels of GGT and other related enzymes. In relation to this point, Hill and Burk (1982) observed that Se deficiency raised plasma GSH plus GSSG level but not cysteine levels in rats.
In addition to increased GSH levels, vit.E and/or Se deficiency has resulted in significantly higher activity levels of GST in liver tissue (Fig. 25A). This observation is consistent with the results of Chang et al., (1990) and Christensen et al., (1994) where elevated GSTs were reported in response to Se deficiency. GSTs, are multifunctional proteins known mainly for detoxification of xenobiotics. The induction of GSTs in response to vit.E and Se deficiency indicates their possible role in antioxidant defenses also. GSTs of a family are known for their role in the reduction of organic peroxides (Chang et al., 1990). It will be interesting to probe further on the specific isozymes of GSTs induced in response to nutritional deficiency and their involvement in antioxidant defenses.

Antioxidants and glutathione related enzymes play a significant role in the defense responses of the lung tissue to oxidant stress (Panus et al., 1988). Se-GSH Px activities were decreased in Se deficient tissues (Fig. 19B) which was also evidenced by studies of Forman et al (1983) and Jenkinson et al (1989). Se deficient lung has reduced activity of Se-GSH Px and, hence, limited capacity to metabolize \( \text{H}_2\text{O}_2 \) and other peroxides. GSH exported from liver to the blood plasma is utilized by the lung, which like liver and kidney exhibits a high overall rate of GSH turnover. In the present study, Se and vit.E deficiency increased GGT activity of lung tissue (Fig. 24B). Activated GGT of lung tissue facilitates the import of substrates required for GSH synthesis. Exercise training is known to increase GGT activities of lung and muscles in rat, but decrease in liver (Sen et al., 1992). During deficiency when hepatic efflux of GSH is accelerated, the lung tissue enjoys a greater ability to enrich its GSH-dependent antioxidant and detoxicant status.

Se and/or vit.E deficiency in the present study enhanced GCS activity of lung tissue (Fig. 22B). This effect was more pronounced in -E-Se animal tissues, which should result in higher GSH levels. On the contrary, GSH levels decreased in lung tissues of -E-Se animals (Fig. 20B). Lungs from Se deficient rats were shown to release glutathione in response to oxidative stress (Jenkinson et al., 1989).
Mice treated with buthionine sulfoxamine (BSO) reduce total lung GSH levels and thereby increasing the susceptibility to oxygen-induced lung damage (Smith and Anderson, 1992). Several lung disorders are believed to be characterized by an increase in alveolar oxidant burden, potentially depleting GSH levels in lung tissue (Martensson et al., 1989). Low GSH has been linked to abnormalities in the lung surfactant system and the interaction between GSH and antiproteases in the epithelial lining fluid of patients. In newborn rats and in adult mice GSH deficiency results in extensive damage to lung type 2 cells with decreased number of lamellar bodies and decreased amounts of intraalveolar tubular myelin (Martensson et al., 1989, 1991). Thus when there is a marked decrease in cellular GSH, the normal physiological formation of reactive oxygen species is unopposed, and this leads to severe cellular damage.

The decreased GSH during vit.E and/or Se deficiency might be due to increased GSH utilization by enzymes such as GSTs, which are induced (Fig. 25B). The rate of depletion by these electrophilic agents is related to the level of GST activity in the cell. Thus GSSG levels were increased which is an indication of oxidative stress. The relatively high concentrations of GSSG may be due to concomitant increase in pulmonary GSTs and increased levels of peroxides in deficient tissues (Fig.22B). Jaeckson and Veal (1990) and Reuter and Klinger (1992) found a marked increase in GSSG levels of lung tissue after hypoxia and reoxygenation. Thus if the rate of GSH consumption exceeds that of import, a tissue may show a net deficit of GSH. The glutathione reductase activities were higher in the lung tissue of vit.E and/or Se deficient animals compared to that of supplemented animals, more pronounced in -E-Se lung tissue to recycle GSH (Fig.23B). Glutathione reductase levels, however, were lower in animals supplied with vit.E and Se compared to deficient animals, indicating the secondary role played by thiols in antioxidant defense mechanisms of lung tissue in the presence of vit.E and Se. Thus it is clear that vit.E and Se deficiency results in inducing oxidant stress in the lung tissue, which is being tackled mainly by cellular thiols. In the present study the observed increase in the levels of GST activities in nutrient
deficient animals suggests a compensatory role for glutathione in protecting hepatic and pulmonary tissues from the oxidative damage induced by vit.E and/or Se deficiency (Fig. 25B).

The present study therefore, reveals that lung tissue is relatively more susceptible to oxidative damage induced by vit.E and/or Se deficiency. As a result of induced oxidative stress, liver and lung tissues of vit.E and/or Se deficient animals showed induction of GSTs. Further studies, however, are required to identify the specific isozyme(s) of GST involved in antioxidant defenses.