ASSESSING THE OXIDATIVE STATUS OF INDIVIDUALS WITH THE METABOLIC SYNDROME - BEFORE, DURING AND AFTER THE CONSUMPTION OF MILLED FLAXSEED OR FLAXSEED OIL

BY

TEJAL PATEL

A thesis submitted to

The Faculty of Graduate Studies

In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Human Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba, Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

Assessing the Oxidative Status of Individuals with the Metabolic Syndrome - Before, During and After the Consumption of Milled Flaxseed or Flaxseed Oil

BY

Tejal Patel

A Thesis/Practicum submitted to the Faculty of Gradute Studies of the University of Manitoba in partial fulfillment of the requirement of the degree of

MASTER OF SCIENCE

ABSTRACT

The global prevalence of Type 2 Diabetes Mellitus (T2DM) has increased exponentially over the last few decades. In Canada, the prevalence of T2DM was 7.2% in 1995 and is estimated to be 9.2% by 2025.

Diabetes mellitus is associated with an increased flux of reactive oxygen species and an alteration in the cellular redox system that results in a loss of reducing capacity, with repercussion on antioxidant defense system. The human body has a complex antioxidant defense system that includes the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) along with the non-enzymatic antioxidants like Vitamin E, C, A, flavanoids and β-carotene.

Flaxseed is one of the richest sources of α-linolenic acid (ALA-52%), and the lignan, secoisolariciresinol diglucoside (SDG-0.6-1.8%), which has the antioxidant activity of inhibiting lipid peroxidation and scavenging produced free radicals specially hydroxyl radical. The effect of consuming flaxseed and flaxseed oil in people with T2DM has not been studied.

Subjects were recruited in accordance with the initial objective of the study of assessing the oxidative status among individuals having T2DM, after consuming milled flax and flax oil in food products. Parameters like their fasting blood glucose and glycated haemoglobin did not support that these subjects had T2DM during the study. Additionally other parameters like lipid profile, waist to hip ratio and BMI, supported that these subjects had metabolic syndrome according ot the definition of the WHO (World Health Organization) and NCEP:ATP III (National Cholesterol Education

Program: Adult Treatment Panel III). Metabolic syndrome is characterized by an aggregation of metabolic risk factors present in an individual putting them at an increased risk of developing pathologic conditions including diabetes mellitus.

Biochemical parameters measured to assess oxidative status were plasma antioxidants Vitamins A (retinol) & E by HPLC, and Vitamin C by Omey's spectrophotometeric assay. Total plasma antioxidant property was determined by the Ferric reducing antioxidant property assay (FRAP); plasma hydroperoxides were analyzed by FOX 2 assay and urinary isoprostanes were estimated with kits from Caymen Chemicals. CAT and SOD activity were determined by the assay described by Aebi and Spitz, respectively, using a UV spectrophotometer.

Results revealed no significant changes among the parameters measured for the different study groups. The antioxidants Vitamins A, E & C were unaltered and FRAP values were in the normal range of healthy adults for all the groups. No significant difference was observed in plasma hydroperoxide and urinary isoprostane levels between the groups consuming either milled flax or flax oil. There was no effect of flaxseed supplementation on erythrocyte SOD or CAT activity.

In conclusion, flaxseed supplementation to subjects with metabolic syndrome showed no effect on oxidative status. Flaxseed did not increase or decrease the indices of lipid peroxidation in these subjects. Hence, focus should be directed on the possibility that flaxseed may be important or effective in interrupting the pathologic process rather than being given when there is no pathologic condition present.

ACKNOWLEDGEMENTS

I would like to thank with all the gratitude to my advisor Dr. James Friel who helped me through all the rough and good times in my project and for providing financial support as well. I am grateful to Dr. Carla Taylor, without her support this project was not possible; she was always there whenever I needed her guidance and thank her for being in my graduate committee.

I am also thankful to Dr. Brian Hasinoff for being in my committee; his inputs were valuable. My gratitude also goes to Dr. Kevin Cockell at University of Toronto for providing the data on protein carbonyls.

Sincere thanks to my previous and current lab members: Paulyne, Soheila, Russel, Apollo and Carla. I also appreciate help provided by HNS department technicians: Marilyn Latta and Dennis Labossiere. I cannot overlook the efforts made by my friend Anita Lloyd, who helped me by all means to carry out statistics on the data obtained.

I deeply appreciate all the efforts by my parents, sister and brother, too numerous to quote, for providing all their support, confidence, strength and encouragement when I needed it the most. I cannot miss upon my roommates and friends, Krutika and Phalguni who were always with me through thick and thin while completing my project. I am grateful to Gitan, Robyn and Betty Ann for guiding me through all the immigration and academic formalities, respectively.

Last but not the least I would like to thank CIHR, Flax Council of Canada and University of Manitoba for supporting my research.

TABLE OF CONTENTS

Page No.

Page No.

LIST OF TABLES

LIST OF FIGURES

Page No.

LIST OF APPENDICES

Page No.

LIST OF ABBREVIATIONS

AGE's	Advanced glycation endproducts
ALA	α- Linolenic acid
AA	Arachidonic acid
ASC	Ascorbic acid
AVD	Atherosclerotic vascular disease
BMI	Body Mass Index
Ca/Mg ATPase	Calcium magnesium adenosine triphosphatase
CAR	Caratenoids
CAT	Catalase
C-H	Carbon hydrogen bond
CHO	Carbohydrate
Cu/Zn SOD	Copper zinc superoxide dismutase
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
GSH	Reduced glutathione
GPx	Glutathione peroxidase
HDL	High density lipoprotein
HOCl	Hypochlorous acid
H_2O_2	Hydrogen peroxide
$HOO^{\cdot}$	Hydroperoxyl radical
LDL	Low density lipoprotein
$^{\cdot}OH$	Hydroxyl radical
Mn	Manganese
MUFA	Monounsaturated fatty acid
Na/K ATPase	Sodium potassium adenosine triphosphatase

$NADP^+$	Nicotinamide adenosine dinucleotide phosphate
$NO^{\cdot}$	Nitric oxide radical
O_2	Molecular oxygen
$O_2^{\cdot}$	Superoxide radical
$OH^{\cdot}$	Hydroxyl radical
PUFA	Polyunsaturated fatty acid
RNS	Reactive nitrogen species
$RO^{\cdot}$	Alkoxyl
$ROO^{\cdot}$	Peroxyl
ROS	Reactive oxygen species
SDG	Secoisolariciresinol diglucoside
SOD	Superoxide dismutase
TG	Triglycerides
TBARS	Thiobarbituric acid reactive substances
TOC	Tocopherol
VLDL	Very low density lipoprotein

CHAPTER ONE – INTRODUCTION

Flax is an economically important oilseed crop especially for Canada. Canada produces about 40% of the world's flaxseed and is the world's largest exporter of flaxseed, representing about 75% of the global flax trade (Oomah, 2001).

Flax supplement to the normal diet may assist in reducing the incidence of diabetes and obesity. Flaxseed is a rich source of α-linolenic acid (ALA), fiber and lignan. Its consumption as a supplement may continue to rise as a result of reported potential beneficial health effects. Some of these include hypocholesterolemic - hypotriglyceridemic effects, a protective effect on colon carcinogenesis and mammary tumors, blood glucose lowering effects and as an anti-inflammatory agent against lupus nephritis (Babu, 2000).

Overall, the prevalence of type 2 diabetes (T2DM) in Canada was 7.2% in 1995 and is estimated to be 9.2% in the year 2025. The etiology of T2DM is uncertain. T2DM is characterized by hyperglycemia, impaired glucose tolerance, insulin resistance and hyperlipidemia. Previous research has suggested that type 1 diabetes mellitus may be due to oxidative stress. The role of oxidative stress in T2DM is not known (Prasad, 2001).

In diabetes mellitus, chronic hyperglycemia produces multiple biochemical sequels, and diabetes-induced oxidative stress could play a role in the onset and progression of the disease (Gallan *et al.,* 2003). Reactive oxygen species are thought to play a role in a variety of physiologic and pathophysiological processes in which increased oxidative stress may play an important role in disease mechanisms; however, increased oxidative stress may be a result of the pathologic process (Gallan *et al.,* 2003).

Evidence indicates that hyperglycemia may disrupt natural antioxidant defense mechanisms. Several experimental studies showing a decrease in the occurrence of complications after supplementation with different antioxidants in diabetic animal models supports the role of lipid peroxidation in the development of diabetic complications (Gallan *et al.,* 2003).

Results from several studies indicate that flaxseed has antioxidative properties and is effective in preventing or delaying occurance of metabolic abnormalities. However, the effect of flaxseed consumption has not been studied among individuals with T2DM. Hence, the present study was planned to determine the effects of milled flaxseed consumption on the oxidative status of subjects with T2DM.

CHAPTER TWO - REVIEW OF LITERATURE

2.1 Type 2 Diabetes Mellitus:

Diabetes is a disease of carbohydrate and lipid metabolism whose hallmark is high blood glucose. T2DM is a complex medical disorder characterized by insulin (hormone that controls blood glucose) resistance and defects in insulin secretion (Quinn, 2002). Insulin is released by pancreatic β-cells primarily in response to elevations in plasma glucose and in response to amino acids such as arginine and lysine. Normal glucose homeostasis is maintained in all physiological conditions by intricately balanced hormones insulin and glucagon. Insulin stimulates glucose uptake, utilization, and storage while suppressing hepatic glucose production and failure to do so results in rise of glucose levels (Quinn, 2002). High blood sugar reflects insulin resistance, meaning the hormone insulin is less effective in assisting the uptake of sugar and fatty acids by muscle and adipose tissues. Glucagon causes the release of newly synthesized or stored glucose and is secreted by the α-cells of the pancreas in response to decreased blood glucose levels (Quinn, 2002). Common symptoms of undiagnosed type 1 diabetes mellitus are frequent urination, frequent thirst and weight loss. In T2DM and other insulin-resistant states, there is an increased flux of free fatty acids into the circulation that results in an increased hepatic production of very low density lipoprotein (VLDL) and causes hypertriglyceridemia (Quinn, 2002). As diabetes progresses, the pancreas loses its ability to produce insulin and this leads to deterioration of other tissues, including the development of circulatory problems, hypertension, kidney disease, impaired regulation of blood clotting, retinopathy, and above all, heart disease.

Diabetes is associated with a variety of metabolic abnormalities and principle among them is hyperglycaemia. Insulin resistance is also an primary risk factor for T2DM. Other risk factors operating at the cellular level to initiate and promote progression of diabetic vascular disease include hyperinsulinemia, altered fatty acid metabolism, hypertension, ketoacidosis, osmotic effects, vasoactive hormones and dysfunction in sympathetic regulation of glucose and fat metabolism (Jakus, 2000).

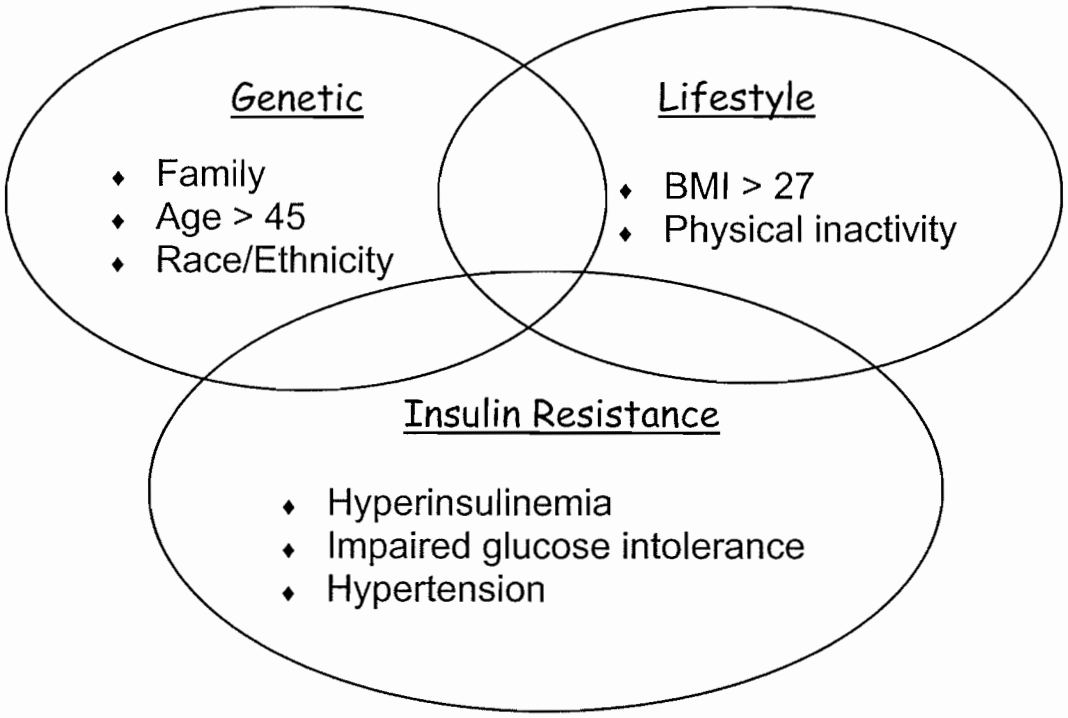
T2DM is a multigenic, multifactorial disorder and several environmental factors contribute to insulin resistance (Simopoulos, 1999). Patients with type 1 or 2 diabetes have an increased risk for micro-macro vascular disease, such as retinopathy, nephropathy, coronary heart disease and diminished cerebral blood supply. There is increasing evidence that an additional load of reactive oxygen species is formed in diabetes via glycoxidation, a process probably relevant at elevated glucose blood levels (Stahl & Sies, 1997).

2.1.1 Risk Factors For Type 2 Diabetes Mellitus:

Genetic, environmental, and metabolic risk factors are interrelated and contribute to the development of T2DM (Fig. 1). A strong family history of diabetes mellitus, age, obesity, and physical inactivity identify those individuals at highest risk. Insulin resistance is a defect in the ability of insulin to take up glucose into the muscle cell. Despite being genetically determined, it is greatly aggravated by obesity and physical inactivity. During the initial phases of insulin resistance, the pancreatic beta cells are able to maintain normal glycemic control through an increased production of insulin, thereby making these individuals hyperinsulinemic. However, when individuals can no

longer maintain normal glycemia from this compensatory mechanism, they progress to the development of T2DM (Fletcher *et al.*, 2002).

Insulin resistance increases a person's risk for developing impaired glucose tolerance and T2DM and risk factors associated with these abnormalities includes hyperinsulinemia, atherogenic dyslipidemia, glucose intolerance, hypertension, prothrombic state, hyperuricemia, and polycystic ovary syndrome.



(Modified from Fletcher *et al.*, 2002)

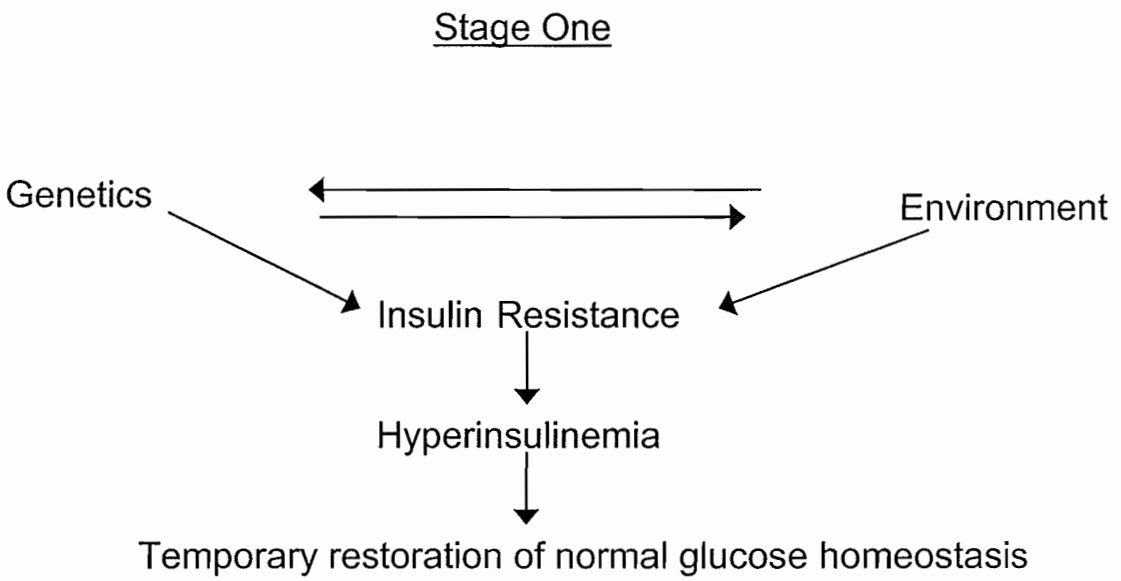
Figure 1: Risk factors for developing Type 2 Diabetes Mellitus and the metabolic abnormalities associated with insulin resistance.

2.1.2 Sequential development of Type 2 Diabetes Mellitus:

Genetic factors likely influence both insulin sensitivity and insulin secretion. In addition, there are usually environmental factors associated with the development of insulin resistance, such as obesity and physical inactivity (Fig. 2). During initial hyperinsulinemia, pancreatic beta cells are able to overcome insulin resistance and maintain normal glucose tolerance by producing a high level of insulin which maintains normal glucose homeostasis (by compensatory hyperinsulinemia).

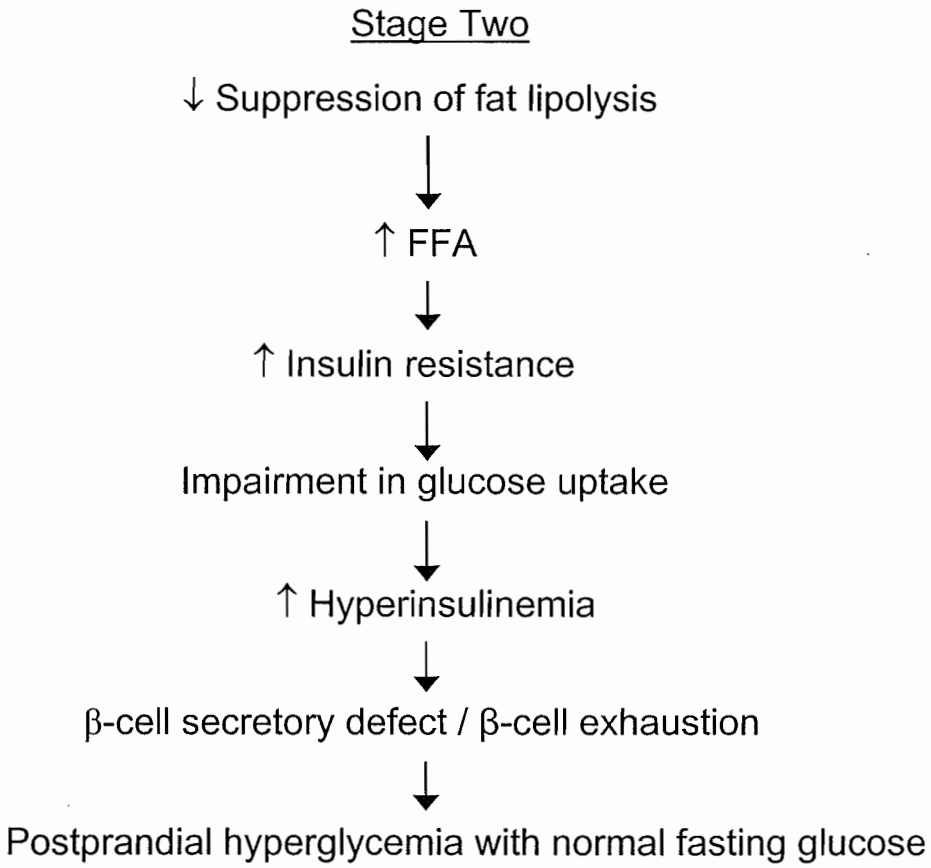
In the second stage (Fig. 3), insulin resistance increases and this compensatory hyperinsulinemia becomes insufficient to maintain normal glucose homeostasis. Insulin resistance in visceral fat leads to increased fatty acid production, exacerbating insulin resistance in liver and muscle. Insulin-mediated glucose transport into skeletal muscle becomes impaired. Fasting plasma glucose levels remain normal but postprandial plasma glucose levels rise.

In the third stage (Fig. 4), there is a further increase in insulin resistance. The restraining effects of insulin on hepatic glucose production become impaired and plasma glucose levels increase. In addition, there are toxic effects of worsening hyperglycemia on the pancreatic beta cell and insulin secretion subsequently declines. With increasing insulin resistance, fatty acids are no longer restrained. The increase in free fatty acids causes a further increase in insulin resistance. Fasting and postprandial hyperglycemia result from increased insulin resistance, unrestrained hepatic glucose production, and glucose toxicity.



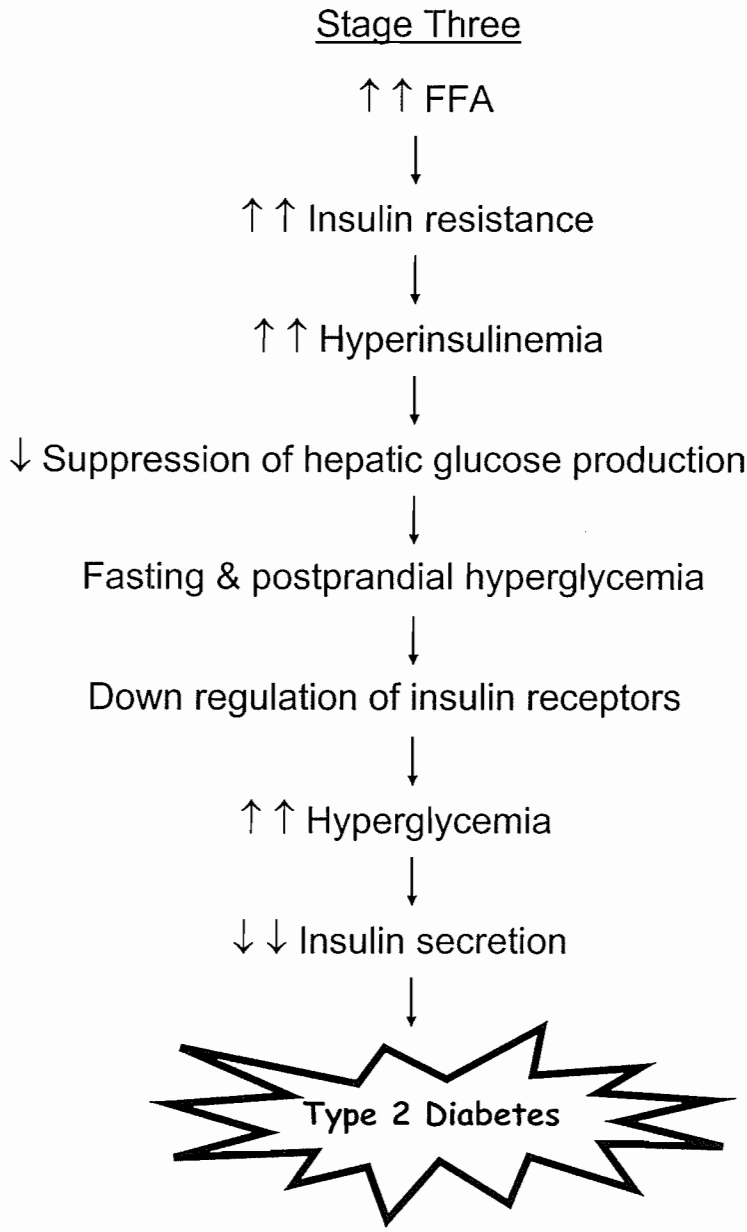
(Modified from Quinn, 2002)

Figure 2: Pathogenesis of Type 2 Diabetes (stage 1)



(Modified from Quinn, 2002)

Figure 3: Pathogenesis of Type 2 Diabetes (stage 2)



(Modified from Quinn, 2002)

Figure 4: Pathogenesis of Type 2 Diabetes (stage 3)

2.2 Production of Reactive Oxygen Species:

2.2.1 Free Radicals and oxidative stress:

Oxygen free radicals and other reactive oxygen species are constantly produced in the human body by unavoidable spontaneous chemical reactions, e.g. production of bursts of reactive species by activated phagocytes in order to help kill invading microorganisms (Halliwell, 2002).

Numerous studies have shown that while different indices of free radical damage increase, there is a decrease in the concentration of various individual antioxidant substances, indicating the presence of oxidative stress in diabetes (Opara, 1999). It has also been suggested that there is a link between the development of microvascular and macrovascular diabetic complications and oxygen free radical damage (Opara, 1999).

2.2.1.1 Free Radicals:

Free radicals are defined as species that contain an odd number of electrons (one or more unpaired electrons); they may be positively charged, negatively charged, or neutral. Reactive oxygen metabolites can be defined as any compound derived from molecular oxygen (O_2) which has acquired (by chemical reduction) less than four electrons (when a compound is reduced, it accepts electrons, whereas when a compound is oxidized, it loses electrons). Free radicals are generally considered to be rather reactive as they will attempt to abstract electrons from other organic substrates in order to pair their odd electrons i.e. gain stability (Grisham, 1994). The oxidized donor molecule then has the capacity to oxidize other molecules, and thus set up a chain reaction that is potentially damaging to surrounding tissues.

Under physiological conditions, damage due to free radicals is countered by antioxidants. Oxidative stress occurs when there is imbalance between free radical reactions and the scavenging capacity of antioxidative defense mechanism of the organism (Jakus, 2000). Thus, oxidative stress is a general term used to describe a state of potential oxidative damage caused by free radicals.

By far, the most common free radicals formed in the body are oxygen-derived and are therefore also known as reactive oxygen species (ROS). These include superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), peroxyl ($ROO^{\cdot}$), alkoxyl ($RO^{\cdot}$) and hypochlorous acid (HOCl) (Jakus, 2000). Other non-oxygen species existing as reactive nitrogen species (RNS) like nitric oxide (NO) and peroxynitrite also have important bioactivity. All can damage proteins through crosslinking, fragmentation and cause lipid oxidation (Jakus, 2000).

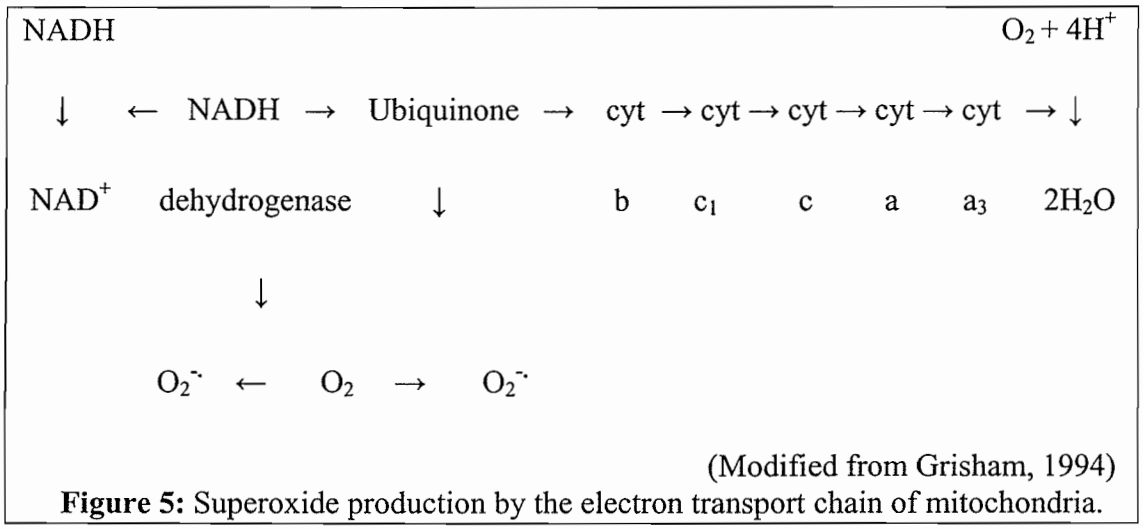
Normally greater than 95% of all O_2 consumed by cells is reduced by four electrons to yield two molecules of water (H_2O) via the mitochondrial electron transport. The enzyme responsible for this reaction is cytochrome oxidase (cytochrome a & a_3). It has been estimated that approximately 1-2% of the electron flow "leaks" off onto O_2 to form $O_2^{\cdot-}$ under normoxic condition. The fact that only H_2O_2 and not $O_2^{\cdot-}$ can be detected using intact mitochondria suggests that $O_2^{\cdot-}$ enzymatically or non enzymatically dismutates to yield H_2O_2 and O_2.

There are two sources of O_2 within the electron transport chain (Fig. 5):

1. The partial reduction of ubiquinone during electron transport. Ubisemiquinone interacts with O_2 to yield $O_2^{\cdot-}$ by the reaction:

$$\text{Ubiquinone} + e^- \rightarrow \text{ubisemiquinone} + O_2 \rightarrow \text{ubiquinone} + O_2^{\cdot}$$

2. The flavin moiety of flavoprotein NADH dehydrogenase enzyme is reduced during catalysis to yield the flavin semiquinone free radical which interacts with O_2 to yield $O_2^{-\cdot}$ via a reaction similar to that described above.



(Modified from Grisham, 1994)

Figure 5: Superoxide production by the electron transport chain of mitochondria.

Superoxide radical is relatively an unstable free radical which exists in equilibrium with its conjugate acid, the hydroperoxyl radical ($HOO^{\cdot}$). The univalent reduction of molecular oxygen yields the superoxide anion radical (Grisham, 1994).

$$O_2^{-\cdot} \leftrightarrow HOO^{\cdot}$$

In aqueous environment, $O_2^{-\cdot}$ will rapidly and spontaneously dismutate to yield H_2O_2 and O_2. A schematic representation showing the sites of $O_2^{-\cdot}$ formation in the electron transport chain is illustrated in figure 5.

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

Hence, the formation of O_2 will always be accompanied by the formation of significant amounts of H_2O_2. Superoxide radical acts as both oxidant and reductant. It will oxidize a variety of biomolecules such as ascorbate, sulfhydryl containing compounds, sulfite and

certain catecholamines. Superoxide can reduce ferric iron chelates and hemoproteins as well as certain quinones (Grisham, 1994).

Hydrogen Peroxide inside the cell will react with certain transition metals such as iron or copper or with certain hemoproteins to yield highly reactive free radicals such as ·OH or ferryl derivatives of hemoproteins. It can inactivate some enzymes by oxidizing essential sulfhydryl groups (Grisham, 1994).

$$O_2 + Fe^{3+} \rightarrow O_2^{-\cdot} + Fe^{2+}$$

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

The hydroxyl radical is the most powerful radical formed which reacts instantly with any neighboring biomolecule.

Peroxyl Radical is produced as intermediates when hydroxyl radicals interact with certain carbohydrates, proteins, nucleotide bases and lipids. Peroxyl radical mediated reactions includes the peroxidation of polyunsaturated fatty acids initiated by ·OH.

$$^{\cdot}OH + LH \rightarrow HOH + L^{\cdot}$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$$

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$$

Where ·OH, LH, L·, LOO· and LOOH represent the hydroxyl radical, polyunsaturated lipid, lipid alkyl radical, lipid hydroperoxyl radical and lipid hydroperoxide, respectively. Peroxyl radical will also oxidize proteins, carbohydrates and sulfhydryl components and hemolyze erythrocytes (Grisham, 1994).

2.2.2 Lipid Peroxidation

The mechanism of lipid peroxidation can be divided into three stages: initiation, propagation and termination. In the presence of initiators (I), unsaturated lipids (LH) lose a hydrogen radical ($H^{\cdot}$) from a weakened C-H bond on carbon atoms adjacent to double bonds in a PUFA to form lipid carbon centered free radicals ($L^{\cdot}$).

$$LH \xrightarrow{I} LH + L^{\cdot}$$

The most likely initiation process is the metal catalysed decomposition of preformed hydroperoxides.

$$LOOH \rightarrow LO^{\cdot} + {}^{\cdot}OH$$

The alkyl radicals of unsaturated lipids ($L^{\cdot}$) contain labile hydrogen, which reacts rapidly with molecular oxygen to form peroxyl radicals. This is the most widely occurring oxidation and describes the first stage of peroxidation of unsaturated lipids producing hydroperoxides as the fundamental primary products.

$$L^{\cdot} + O \rightarrow LOO^{\cdot}$$

At the last stages of oxidation, after reaching a maximum, the rate decreases. The peroxyl radicals then react with each other and self destruct to form non-radical products by the termination reaction which occurs by the combination of peroxyl radicals to an unstable tetroxide intermediate followed rapidly by its decomposition yielding non radical products (Frankel, 1998).

2.3 Diabetes is accompanied by severe oxidative stress:

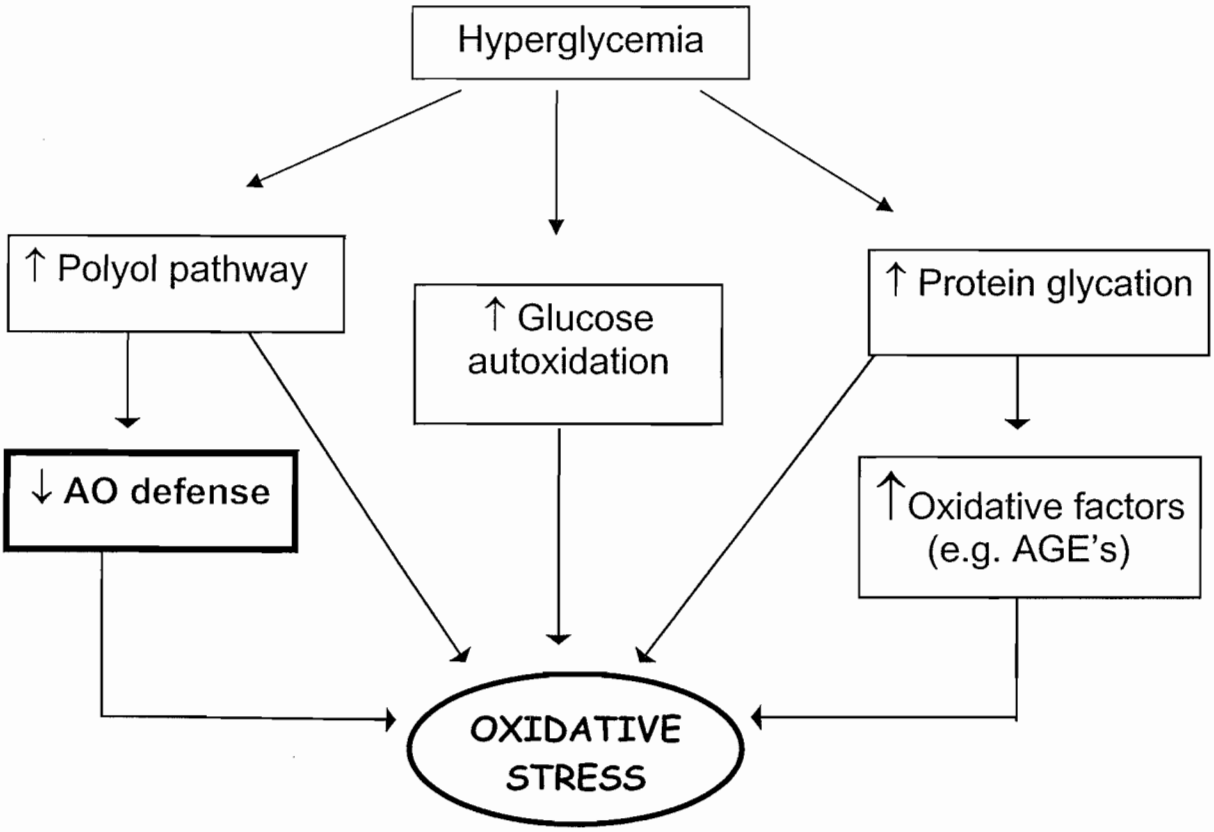
There are many ways by which hyperglycemia increases the generation of free radicals. Chronic hyperglycemia in diabetes enhances the production of ROS from glucose autooxidation, protein glycation and the polyol pathway (Fig. 6).

The term autoxidation describes the capability of glucose to enolize, thereby reducing molecular oxygen and yielding oxidizing intermediates (Giugliano *et al.,* 1996). The reduced oxygen products formed in autoxidative reactions can damage lipids, as well as proteins, through cross-linking and fragmentation.

Protein Glycation: One of the mechanisms linking hyperglycemia to the vascular complications of T2DM is the non-enzymatic glycosylation of proteins. The process of glycosylation begins with the attachment of glucose to the amino group (of the lysine residue of protein) which undergoes a series of chemical modifications and produces insoluble complexes termed advanced glycation endproducts (AGE's) which are increased in hyperglycemia.

Glucose autoxidation: Autoxidation of glucose leads to hydrogen peroxide, ROS and reactive ketoaldehydes, which modify the cellular proteins leading to their fragmentation by free radical mechanism. Free radicals also accelerate the formation of advanced glycosylation end products which in turn supplies more free radicals. This process is termed autoxidative glycosylation or glycoxidation (Giugliano *et al.,* 1996).

Polyol Pathway: Glucose is metabolized to sorbitol. The rate-limiting enzymes involved in the conversion of glucose to sorbitol are aldose reductase and sorbitol dehydrogenase. Accumulation of sorbitol (decrease in myoinositol) leads to changes in membrane functions e.g. decrease in antioxidants (AO), osmotic swelling,



(Modified from Giugliano *et al.*, 1996)

Figure 6: Possible links between hyperglycemia and oxidative stress.

cellular destruction, reduced Na K ATPase activity and the failure of this pump.

Some nonenzymatic sources of reactive oxygen metabolites includes following autoxidation reactions (Grisham, 1994):

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-\cdot}$$

$$\text{Haemoglobin–}\ Fe^{2+} + O_2 \rightarrow \text{Haemoglobin} - Fe^{3+} + O_2^{-\cdot}$$

$$\text{Myoglobin -}\ Fe^{2+} + O_2 \rightarrow \text{Myoglobin–}\ Fe^{3+} + O_2^{-\cdot}$$

$$\text{Catecholamines} + O_2 \rightarrow \text{Melanin} + O_2^{-\cdot}$$

$$\text{Reduced flavin} + O_2 \rightarrow \text{Flavin semiquinone} + O_2^{-\cdot}$$

$$\text{Coenzyme Q} + O_2 \rightarrow \text{Coenzyme Q} + O_2^{-\cdot}$$

$$\text{Tetrahydroprotein} + 2O_2 \rightarrow \text{Dihydroprotein} + 2\ O_2^{-\cdot}$$

Multiple studies have shown that T2DM is accompanied by increased oxidative damage to all biomolecules, especially lipids (Halliwell, 2002). Hyperglycemia has been shown to increase oxygen free radical production in vascular endothelial cells, nervous tissue, the rodent embryo and human monocytes. Lipid peroxidation may be increased in insulin-resistant individuals well before the onset of T2DM. In other words, not only do sequelae of T2DM cause elevated peroxidation, but also pre-existing high rates of lipid peroxidation may predispose to diabetes (Halliwell, 2002).

In diabetes, oxidative stress seems caused by both increased production of ROS, a sharp reduction in antioxidants defenses and altered cellular redox status. Patients with diabetes may be especially prone to acute and chronic oxidative stress, which enhances development of late diabetic complications (Jakus, 2000).

There is evidence that acute elevations in glucose levels may depress natural antioxidants defenses e.g. incubation of purified bovine CuZn-SOD with different glucose concentrations (10-100 Mmol/L) reduced the enzymatic activity by 60% (Giugliano *et al.,* 1996).

Therefore, not only the diabetic patients are exposed to ROS but the diabetic patients also have a compromised defense system.

2.3.1 Protein Carbonyls:

In the last 2 years, attention has focused on the role of carbonyl stress in diabetes. Under oxidative stress, carbohydrates and lipids, as well as proteins are the major targets of reactive oxygen species. Reactive carbonyl compounds formed from carbohydrates, lipids, and amino acids by both oxidative and nonoxidative pathways increase in the carbonyl stress. This reactive carbonyl compounds contribute to the formation of AGEs with the initiation of the Maillard reaction, known to be implicated in the pathogenesis of diabetic complications (Gallan *et al.,* 2003). Autoxidation of carbohydrates and ascorbate yields carbonyl compounds e.g. glyoxal, arabinose, methyglyoxal, glycolaldehyde, and dehydroascorbate (Miyata *et al.,* 1998). Lipid peroxidation of polyunsaturated fatty acids, such as arachidonate, yields other carbonyl compounds such as malondialdehyde (MDA) and 4-hydroxy-nonenal (Miyata *et al.,* 1998).

Oxidative damage of proteins is one of the modifications leading to severe failure of biological functions and cell death (Gallan *et al.,* 2003). Oxygen free radicals produced by the metal catalyzed oxidation systems have been assumed as the primary mechanism for the formation of protein carbonyls *in vivo* (Miyata *et al.,* 1998). An overload of ROS

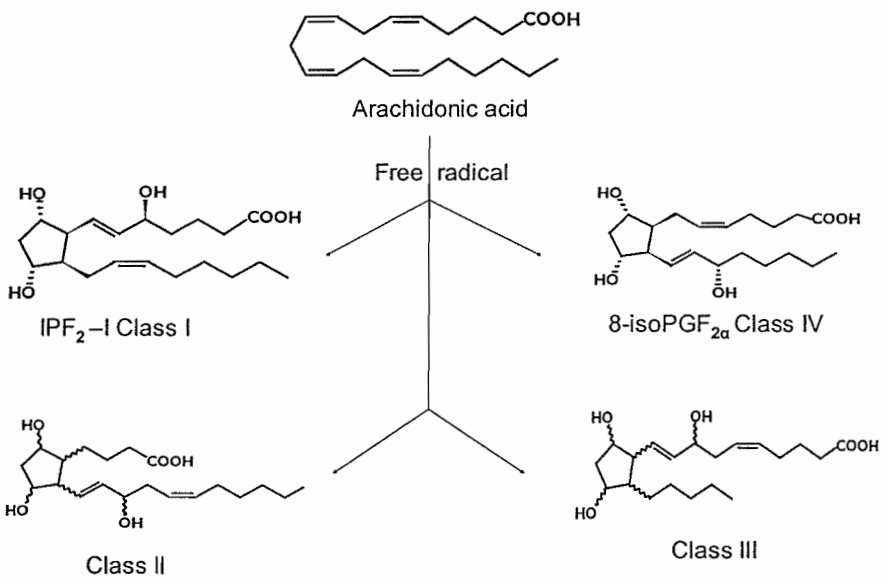
is known to primarily modify proteins and generate reactive aldehydes derived from lipid peroxidation, and these molecules, in turn, can produce secondary modifications of proteins (Gallan *et al.,* 2003). Prolonged exposure of protein to reactive molecules leads to spontaneous postsynthetic modifications, such as glycation or oxidation. Protein carbonyls are considered as a sensitive index of oxidative injury to proteins. Moreover, protein glycation and covalent binding of certain aldehyde end-products of lipid peroxidation to proteins can also generate carbonyls (Gallan *et al.,* 2003). It has been described that various proteins, including haemoglobin, albumin, collagen, LDL, or crystalline proteins, undergo nonenzymatic glycation in diabetes (Gallan *et al.,* 2003). Miyata *et al.,* (1998) also reported that glucose which is the major source of glycation or the Maillard reaction *in vivo*, turned out not to represent the source of protein carbonyls.

2.3.2 Isoprostanes:

The discovery of F_2-isoprostanes (prostaglandin-like compounds), *in vivo* products of nonenzymatic lipid peroxidation of arachidonic acid, has been a major breakthrough regarding the quantification of oxidant stress *in vivo* (Devaraj *et al.,* 2001 and Morrow & Roberts, 2002). F_2-isoprostanes are initially formed esterified to phospholipids and then released in free form. There are several favourable attributes that make measurement of F_2-isoprostanes attractive as a reliable indicator of oxidative stress *in vivo*: (1) F_2-isoprostanes are specific products of lipid peroxidation, (2) they are stable compounds, (3) levels are present in detectable quantities in all normal biological fluids and tissues, (4) their formation increases dramatically *in vivo* in a number of animal

models of oxidant injury, (5) their formation is modulated by antioxidant status and (6) their levels are not affected by lipid content of the diet (Roberts & Morrow, 2000).

Initially, three arachidonoyl radicals are formed which then undergo endocylization to form four prostaglandin H_2-like bicyclic endoperoxide intermediate regioisomers which are reduced to four F-ring regioisomers. Each regioisomer is comprised of eight racemic diastereomers. Because these compounds are isomeric to prostaglandin $F_{2\alpha}$ formed by the cyclooxygenase, they have been termed F_2-isoprostanes (Roberts & Morrow, 2000) (Fig. 7).



(Modified from Patrono & Fitzgerald, 1997)

Figure 7: Four classes of F2 isoprostanes that may be formed by free radical attack on arachidonic acid.

After their formation, isoprostanes are released from the membrane phospholipids in plasma. The factors that regulate release of endogenous isoprostanes from cell membranes and interconversion between the free and esterified forms are presently poorly understood. It is conjecturable that hyperglycemia could influence the release of isoprostanes from cell membrane via increased activation of protein kinase C and phospholipase A_2 (Mezzetti *et al.,* 2000).

Plasma and urinary isoprostanes are increased in T2DM (Sampson *et al.,* 2002 and Devaraj *et al.,* 2001) and there is an indirect relationship to measure chronic hyperglycemia (Sampson *et al.,* 2002). The level of a metabolite in urine collected over many hours can provide an integrated index of isoprostanes production over time (Roberts & Morrow, 2000).

2.3.3 Hyperglycaemia, insulin, insulin resistance and oxidative stress:

Insulin stimulates the uptake and utilization of glucose in muscle and adipose tissue, inhibits glycogenolysis and gluconeogenesis in the liver and inhibits lipolysis in adipose tissue. Hyperglycaemia impairs the physiological homeostasis of many systems in living organisms. Excessive hyperglycaemia may impair insulin activity and sensitivity by the mechanism of glucose toxicity. A deficiency in insulin action reverses the metabolism in the opposite direction (Jakus, 2000). Thus, increased lipolysis enhances the level of free fatty acids and their oxidation in liver. Glucotoxicity (excess glucose) and lipotoxicity (excess fatty acids) could lead to further insulin resistance and hyperinsulinemia (Jakus, 2000).

2.3.4 Oxidative stress in diabetes mellitus:

In diabetes, oxidative stress seems to be caused by both increased production of ROS, a sharp reduction in antioxidant defenses and altered cellular redox status (Jakus, 2000).

Possible sources of oxidative stress in diabetes mellitus (Jakus, 2000):

(A) Increased generation of ROS

- Autoxidation of CHO, autoxidation of fatty acid in TG, phospholipids and cholesteryl esters
- Acute and chronic hyperglycaemia
- Glycation, advanced glycation and glycoxidation.

(B) Decreased antioxidant defense

- Alterations in glutathione concentration or metabolism
- Decrease in antioxidant systems, such as CAT, SOD or GPx
- Alterations in Vitamin E and ascorbate homeostasis
- Alterations in concentrations of other antioxidants, such as ubiquinol, carotene, taurine and uric acid.

(C) Alterations in enzymatic pathways

- Increased polyol pathway activity
- Decreased glyoxalase pathway activity
- Alteration in mitochondrial oxidative metabolism
- Altered prostaglandin and leukotriene metabolism

(D) Other mechanisms

- Ischaemia-reperfusion injury, hypoxia and pseudohypoxia.

2.3.5 Effects of oxidative stress on insulin action:

Increasing evidence suggests that the fatty acid composition of membrane lipids influences the action of insulin in a number of tissues (Paolisso & Giugliano, 1998). Enhanced free radical production can move free radical hydrogen atoms to another polyunsaturated fatty acid, thus generating another free radical and lypohydroperoxide. This produces damage in living cells through 2 mechanisms:

1. mechanical disruption to the membrane causing a loss of the coordinating function of the enzymatic systems contained within it;
2. production of toxic substances from the disintegration of polyunsaturated fatty acids, which are able to migrate from the production sites and reach distant targets.

This production of toxic substances may cause inhibition of a series of enzymatic systems such as microsomal glucose-6-phosphate and plasma membrane Ca/Mg activated ATPase and Na/K activated ATPase pump (Paolisso & Giugliano, 1998).

Studies carried out in type 2 diabetic patients revealed that plasma free radical concentrations were significantly correlated with fasting plasma insulin levels and insulin-mediated glucose uptake (Paolisso & Giugliano, 1998).

Another study carried out by Mattia *et al.,* (2003) revealed that the peroxide levels of the type 2 diabetic patients were significantly higher than healthy individuals (19.45 ± 4.7 Vs 9.5 ± 3.7 M/L). Also total radical trapping capacity was lower in diabetic subjects compared to normal adults (1019.4 ± 180.9 Vs 1326.8 ± 105.2 μM/L). Among the causes of enhanced free radical production, hyperinsulinaemia and hyperglycaemia seem to play a major role (Paolisso & Giugliano, 1998).

2.4. Interrelationship between diabetes and free radicals:

Oxidative stress can be increased before clinical signs of diabetic complications. However, the role of oxidative stress in the initiation and progression of diabetes remains uncertain (Jakus, 2000). Formation of free radicals is associated with Maillard reactions. The maillard reaction is an amplifier of oxidative stress. Glycated proteins produce nearly 50 fold more free radicals than non-glycated proteins. In diabetes, the process of production of superoxide radicals by transition metals catalyses autoxidation, followed by the dismutation of superoxide to hydrogen peroxide. The generation of hydroxyl radicals by Fenton reaction, results in site-specific attacks on protein, with consequent protein damage and lipoxidation and damage to other cell components, such as DNA (Jakus, 2000).

2.5. Antioxidants:

An antioxidant has been defined as "a substance that when present at low concentration compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate" (Packer *et al.,* 2002). The organism maintains defense systems against reactive oxygen species, including enzymes and low-molecular weight antioxidants (Stahl & Sies, 1997).

Reactive oxygen species are continuously produced as metabolic byproducts (in relatively small amounts) by virtually all tissues. All mammalian cells contain a number of different enzymatic and nonenzymatic antioxidants that serve to prevent or limit oxidative tissue injury. The first line of defense against reactive oxygen species are the enzymatic antioxidants including superoxide dismutase, catalase and glutathione

peroxidase. Second line of defence consists of the non-enzymatic antioxidants like Vitamins A, C, E, beta-carotene, flavonoids etc.

2.5.1 Antioxidant property of Vitamins:

Important antioxidants such as Vitamins E and C and carotenoids are provided from the diet. Vitamin E, as the major chain breaking antioxidant, inhibits lipid peroxidation, thus preventing membrane damage and modification of low density lipoproteins. It is regenerated by the water-soluble Vitamin C. Carotenoids efficiently scavenge singlet molecular oxygen and peroxyl radicals (Stahl & Sies, 1997).

2.5.1.1 Antioxidant property of Vitamin C:

Vitamin C (Fig. 8) is one of the most powerful water soluble natural antioxidants found in high concentration in many tissues. Human plasma contains about 60 μM/L ascorbate. Upon interaction with reactive oxygen species, Vitamin C is oxidized to dehydroascorbate via the intermediate ascorbyl free radical. Dehydroascorbate is recycled back to ascorbic acid by dehydroascorbate reductase. As a scavenger of reactive oxygen species, ascorbate has been shown to be effective against superoxide radical anion, hydrogen peroxide, hydroxyl radical and singlet oxygen. In aqueous solutions, Vitamin C also scavenges reactive nitrogen oxide species efficiently preventing the nitrosation of target molecules. Ascorbate can also act as prooxidant *in vivo*. In the presence of free transition metal ions and ascorbate, hydroxyl radical can be generated, and initiation of lipid peroxidation may occur (Stahl & Sies, 1997).

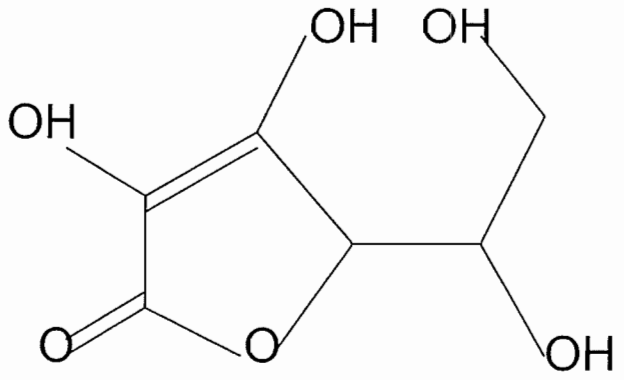


Figure 8: Structure of Vitamin C (ascorbic acid) (Modified from Frenich *et al.,* 2005)

2.5.1.2 Mechanism of Vitamin C against free radical attack:

Several properties make Vitamin C an ideal antioxidant in biological systems. First, the low one electron reduction potentials of ascorbate and the ascorbyl radical enable these compounds to react with and reduce virtually all physiological relevant reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Packer *et al.,* 2002). The ascorbyl radical is neither strongly oxidizing nor strongly reducing and it reacts poorly with oxygen. Thus, when a reactive radical interacts with ascorbate, a much less reactive radical is formed. The ascorbyl radical scavenges another radical or rapidly dismutates to form ascorbate and dehydroascorbic acid (Packer *et al.,* 2002).

$$\text{Ascorbic acid} + 2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + \text{dehydroascorbic acid}$$

A study carried out by Panda (2000) showed that in the absence of ascorbic acid, cigarette smoke caused oxidation of human plasma proteins and extensive degradation of guinea pig lung and heart microsomes.

2.5.1.3 Antioxidant property of Vitamin E:

The term Vitamin E is a generic description for all tocols and tocotrienol derivatives that exhibit the biological activities of α-tocopherol. This group of compounds is lipophilic, operative in membranes or lipoprotein particles. An important antioxidant function appears to be the inhibition of lipid peroxidation, scavenging lipid peroxyl radicals to yield lipid hydroperoxides and the tocopheroxyl radical. The latter is less reactive with neighboring polysaturated fatty acids than are peroxyl radicals and can be either reduced by ascorbate or glutathione or further oxidized to the quinine. Compared with other lipophilic antioxidants α-tocopherol is the most efficient antioxidant in the lipid phase (Stahl & Sies, 1997). The normal plasma tocopherol values have reported to be in range of 5 -16 μg/ml (Farrell *et al.,* 1978).

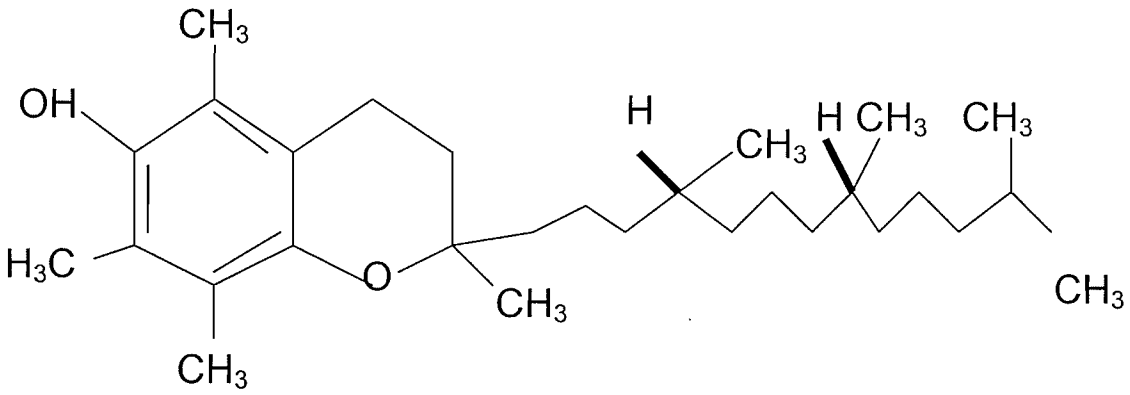


Figure 9: Structure of Vitamin E (α-tocopherol) modified from Al-Talla & Tolley, 2005)

2.5.1.4 Mechanism of Vitamin E against free radicals:

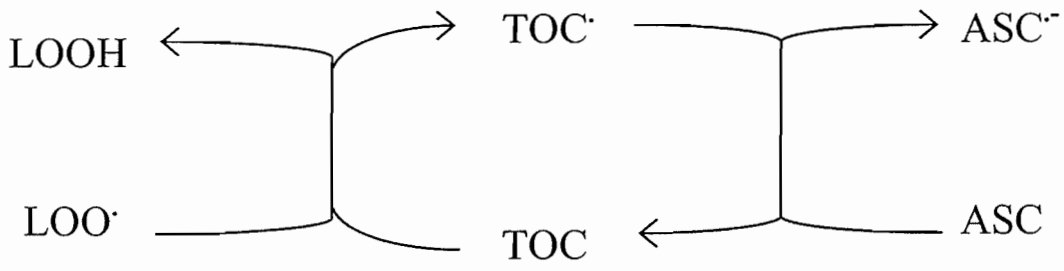
α-Tocopherol (Fig. 9) behaves like a chain breaking electron donor antioxidant by competing with the substrate for the chain carrying peroxyl radicals, normally present in the highest concentration in the lipid system. α-Tocopherol reacts rapidly with peroxyl radicals by donating a hydrogen atom to produce lipid hydroperoxides and a tocopherol radical that is stabilized by resonance. This tocopherol radical does not propagate in the

chain but forms non-radical products, including stable peroxides, which can be reduced to a tocoquinone adduct and to tocopherol dimers. α-Tocopherol is also oxidized to form α-tocopheroquinone, which acts as an electron acceptor antioxidant by competing with oxygen for alkyl radicals. This competitive reaction would only become important at low oxygen pressure, because the alkyl radicals react extremely rapidly with oxygen under atmospheric conditions (Frankel, 1998).

Halliwell (2002) has mentioned that when diabetic type 2 patients were supplemented with Vitamin E for 3 months, there was decrease in lipid peroxidation and free radical production by circulating monocytes. He also mentioned that Vitamin E supplementation has shown improved vascular endothelial function in diabetic patients.

2.5.1.5 Synergistic effect of Vitamin E and Vitamin C:

The synergistic interactions between α-tocopherol and ascorbic acid are mainly explained by the regeneration of the tocopheroxyl radical (produced during oxidation) intermediate to the parent tocopherol by Vitamin C (Frankel, 1998) (Fig. 10). In addition, the low reactivity of ascorbyl radical, which is formed when Vitamin C scavenges ROS or RNS, makes Vitamin C an ideal antioxidant. The ascorbyl radical is neither a strongly oxidizing nor a strongly reducing agent and it reacts poorly with oxygen. Thus, when a reactive radical interacts with ascorbate, a much less reactive radical is formed. The ascorbyl radical scavenges anther radical or raplidy dismutates to form ascorbate or dehydroascorbic acid (Packer *et al.,* 2002).



LOOH=peroxidated lipid
TOC=Tocopherol
ASC=Ascorbate

(Modified from Frankel, 1998)

Figure 10: Synergic interplay of ascorbic acid and tocopherols in the protection of lipids against oxidation.

The key step is the reaction between the tocopheroxyl radical (TOC·) and Vitamin C (ASC):

$$TOC^{\cdot} + ASC^{-} = TOC + ASC^{\cdot}$$

Ascorbic acid cannot inhibit the oxidation of lipids when the radicals are initially generated within the lipid phase where tocopherols are active. However, ascorbic acid is able to interact with tocopheroxyl radicals, which is the basis for the synergistic effects of these two compounds. The synergistic interactions between tocopherols and ascorbic acid may be of importance in inhibiting the initial formation of hydroperoxides accelerated by tocopherols.

2.5.2 Endogenous antioxidant enzyme activities:

Superoxide dismutase present in the cytoplasm contains the copper-zinc SOD which has two molecules of copper and a zinc per dimeric protein. Mitochondria have manganese containing SOD, which functions to remove $O_2^{\cdot -}$ generated by the leak of

electrons from the electron transport chain. Both SOD isozymes catalyze the dismutation of $O_2^{-\cdot}$ to yield H_2O_2 and O_2 (Grisham, 1994) (Fig. 11).

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

Catalase is present primarily in liver and erythrocyte. It is heme-containing metalloenzyme that catalyzes the decomposition of H_2O_2 by the following reaction:

$$\text{Catalase } Fe^{3+} + H_2O_2 \rightarrow \text{Compound I}$$

$$\text{Compound I} + H_2O_2 \rightarrow \text{Catalase } Fe^{3+} + H_2O_2 + O_2$$

In the case of catalase, H_2O_2 acts as both oxidant and reductant. Catalase is present predominantly in the peroxisomes where a number of H_2O_2 generating enzymes are present including amino acid oxidase and uric acid oxidase (Grisham, 1994). Catalase is also localized in lysosomes and mitochondria and is considered to be effective for protection against oxygen radical damage to cytosolic and nuclear compartments, where H_2O_2 can spontaneously convert into the highly reactive $OH^{\cdot}$ radicals in presence of transition metals, primarily Fe^{2+} and Cu^+ (Turk *et al.,* 2002).

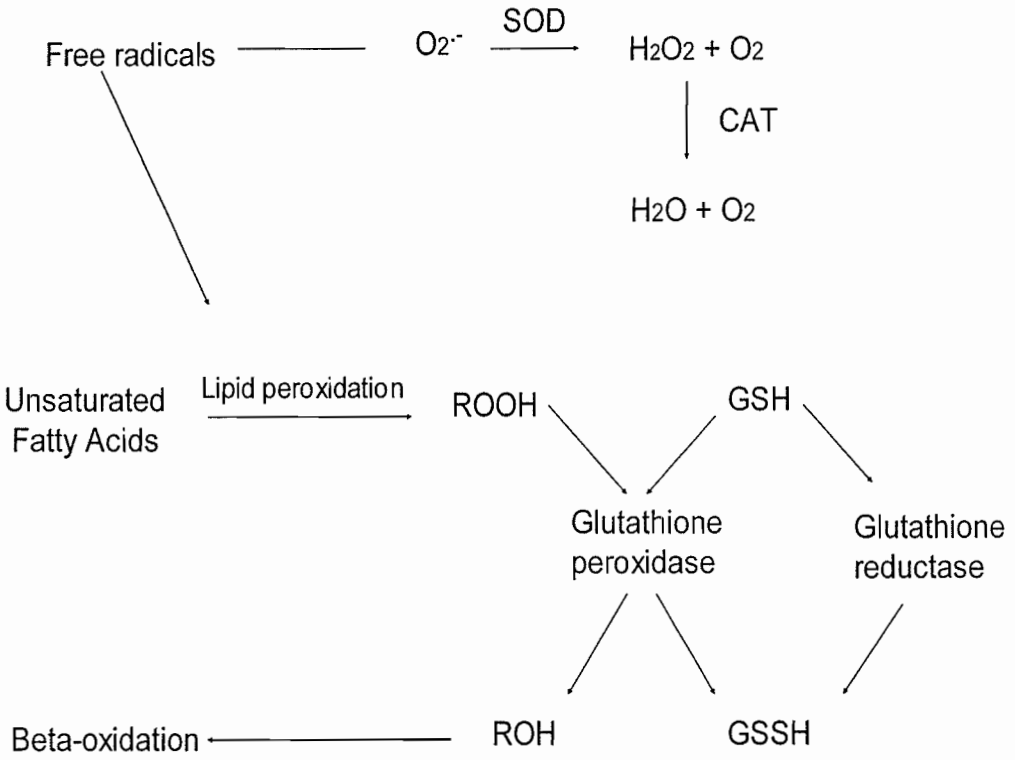
Glutathione peroxidase is an antioxidant enzyme that contains selenium at its active site. It is responsible for the decomposition of peroxides using reduced glutathione as the hydrogen donor (Grisham, 1994).

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

This enzyme has an absolute specificity for GSH as its electron donating substrate; however, its specificity for peroxide is much less selective e.g. GSH peroxidase will utilize a variety of organic peroxides as substrates including H_2O_2, a wide variety of organic peroxides and several different lipid peroxides. Once oxidized, GSH is regenerated from GSSG via the enzyme glutathione reductase (Grisham, 1994).

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

The ratio of GSH and GSSG is maintained at a high level so that the cell maintains the capacity to combat oxidative insult (Grisham, 1994).



(Modified from Parthiban *et al.*, 1995)

Figure 11: Detoxification of superoxide ions and hydrogen peroxide.

2.5.2.1 Antioxidant Status in Diabetes:

Glutathione peroxidase activity in erythrocyte was significantly reduced in diabetic type 1 patients with poor and medium metabolic control in relation to the control group regardless of the existence or absence of vascular disorders. No difference was found in erythrocyte SOD activity between diabetic and control groups (Ruiz, 1999).

A study carried out by Kesavulu *et al.,* (2000) found that erythrocyte catalase activity was increased and glutathione peroxidase activity was decreased in type 2 diabetic patients compared to controls, but there was no significant change in erythrocyte superoxide dismutase activity was found in diabetics. In addition, the diabetic group with microvascular complications had decreased erythrocyte glutathione peroxidase and superoxide dismutase activities compared to the diabetic group without microvascular complications, while no difference was found in erythrocyte catalase activity in both the groups.

Farkas *et al.,* (unpublished data) states that compared to controls, poor metabolic status was associated with a decrease in reduced & oxidized glutathione and higher catalase activity in erythrocyte. Lower erythrocyte glutathione peroxidase and higher erythrocyte catalase activity were observed in diabetic patients with poor metabolic compared to control subjects without complications.

Akkus *et al.,* (1996) reported that leukocyte Vitamin C levels were significantly reduced in patients with T2DM and that leukocyte SOD activity was slightly but not significantly reduced (Control 42.9 ± 17.9 $\mu g/10^8$ leukocyte, 0.45 ± 0.20 U/mg protein; Diabetic 36.5 ± 14.3 $\mu g/10^8$ leukocyte, 0.41 ± 0.19 U/mg protein respectively). However, there was no change in the leukocyte GPx activity and serum Vitamin C levels. There

was no correlation between any of the parameters and fasting blood glucose and glycated haemoglobin. No correlations were found among leukocyte lipid peroxidation, Vitamin C level, SOD and GPx activity.

It has been proposed that poor glycemic control is also associated with the depletion of protective serum antioxidant enzyme activities including superoxide dismutase, catalase and glutathione peroxidase in T2DM (Sozmen *et al.,* 2001). This research group reported an increase in the serum catalase activity and a decrease in the basal and salt stimulated paraoxonase activity of patients compared with controls, and no significant difference in serum SOD activity.

Mehmet *et al.*, (2000) reported that after 2 months of dietary treatment, serum lipid peroxidation decreased and erythrocyte SOD and GPx activities increased in diabetic patients. The diet was composed of 50-55% CHO and 30% fat.

Another study by Turk *et al.,* (2002) showed that plasma TBARS levels and plasma SOD activity were elevated in the type 2 diabetic group when compared with normal controls. However, plasma catalase activity was significantly decreased in the diabetic group when compared with the control group.

A similar study carried out by Memisogullari *et al.*, (2003) suggests that the antioxidant deficiency and excessive peroxide-mediated damage may appear in T2DM. Results showed that levels of erythrocyte lipid peroxidation, serum ceruloplasmin, HbA1c, serum glucose levels and erythrocyte catalase activity were significantly increased, whereas serum albumin, serum transferrin, erythrocyte glutathione, and erythrocyte glutathione peroxidase activity were significantly decreased compared to controls. Erythrocyte superoxide dismutase activity was unchanged.

2.3 Properties of flaxseed:

Flaxseed (*Linum usitatissimum*) is emerging as one of the key sources of phytochemicals in the functional food arena. It is one of the richest sources of the omega 3 fatty acid, α-linolenic acid (ALA-52%), and lignan, secoisolariciresinol diglucoside (SDG 0.6-1.8%). Flaxseed is an essential source of high quality protein and soluble fibre and has considerable potential as a source of phenolic compounds (Oomah, 2001).

2.3.1 Major nutritional components of flaxseed:

Flaxseed oil contains a mix of fatty acids. It is high in PUFA (73%), moderate in MUFA (18%) and low in SFA (9%) (Fig. 12). ALA is an EFA and the parent FA of the omega 3 family. It can be converted to two main long chain fatty acids, eicosapentaenoic acid (EPA) and docohexaenoic acid (DHA) in a series of enzymatic reactions (Fig. 13). Omega 3 fatty acids have been shown to regulate gene transcription and expression, thus altering enzyme synthesis, and to modify several risk factors for coronary heart disease, including reducing serum triglycerides and blood pressure. EFAs are required for maintaining the structure of cell membranes and the permeability of the skin, as precursors for eicosanoids such as prostaglandins and thromboxanes, and in cholesterol transport and metabolism.

Lignans (diphenolic structure formed by joining two cinnamic acid derivatives) are phytoestrogen-compounds found in plants that have weak estrogen activity in animals. Lignans have numerous biological properties, including antimitotic, antifungal and antioxidant activity. The richest source of lignans is flaxseed, which contains high levels of the lignan precursor secoisolariciresinol diglucoside (SDG) and which provide

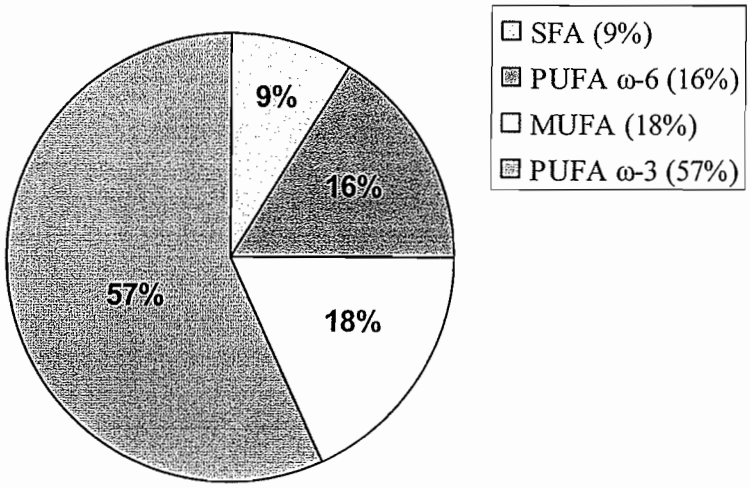
75-800 times more plant lignans than most other foods found in vegetarian diets. Lignans are not present in appreciable amounts in flaxseed oil as most of lignans are removed during processing.

SDG is classified as a phytoestrogen since it is a plant derived nonsteroid compound that possesses estrogen like activity. The level of SDG in flaxseed typically varies between 0.6-1.8%. The flaxseed lignan precursor SDG is converted by gut microorganisms to the main lignans found in humans and other mammals - enterodiol and enterolactone, by a series of hydrolysis, dehydroxylation and demethylation reactions (Fig. 14). Enterodiol and enterolactone have been shown to have antioxidant activity *in vitro* approximately 3 times greater than SDG. They have a number of antioxidant activities including inhibition of lipid peroxidation and scavenging of hydroxyl radicals (Prasad, 1999).

Flaxseed oil is a potent inhibitor of pro-inflammatory mediators. The growing concern that the linoleic acid content of the typical western diet is too high, has lead some experts to recommend replacing dietary omega 6 fatty acid with those from the omega 3 family (Oomah, 2001). A simple addition of flaxseed oil to canola oil in a 1:3 ratio can beneficially mediate the effects of ALA on the eicosanoids, producing a significant reduction in the risk of cardiovascular disease (Oomah, 2001). Consumption of flaxseed either raw or defatted reduces total and LDL cholesterol in humans confirming the multi-component cardioprotective effect of flaxseed (Oomah, 2001).

Fat	41%
Total dietary fibre	28%
Protein	20%
Moisture	7%
Ash	4%

(a)



(b)

(Modified from Flax Council of Canada)

Figure 12: (a) Composition of flaxseed and (b) Proportion of fatty acid in flaxseed oil.

α-linolenic acid ALA (18:3n-3)

↓ Δ 6 desaturase

Stearidonic acid (18:4n-3)

↓ Elongase

Eicosatetraenoic acid (20:4n-3)

↓ Δ 5 desaturase

Eicosapentaenoic acid EPA (20:5n-3)

↓ Elongase

Docosapentaenoic acid (22:5n-3)

↓ Δ 4 desaturase

Docosahexaenoic acid DHA (22:6n-3)

(Modified from Flax Council of Canada)

Figure 13: Metabolic pathways of the omega-3 fatty acids.

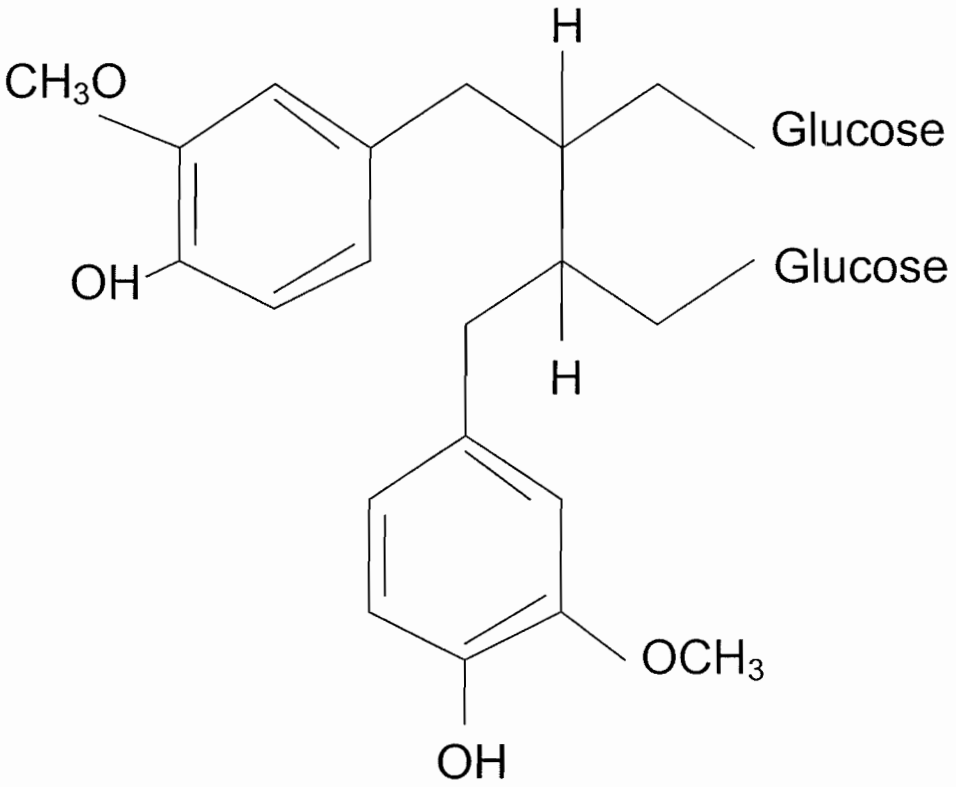
Flaxseed gum has nutritional value as a dietary fibre; it appears to play a role in reducing diabetes and coronary heart disease risk, preventing colon and rectal cancer and reducing the incidence of obesity. It behaves like typical viscous fibres with the ability to flatten the blood glucose profile (Oomah, 2001).

Flaxseed protein is also an excellent source of arginine, glutamine and histidine, the three amino acids known to have a strong effect on the immune functions of the body. The high cysteine and methionine content of flaxseed protein can boost the body's antioxidant levels, potentially stabilizing DNA during cell division and reducing the risk of certain forms of colon cancer (Oomah, 2001).

Dietary supplementation to hyperlipidemic subjects with partially defatted flaxseed (50 gm/day) reduced total cholesterol (4.6 ± 1.2%), LDL-C (7.6 ± 1.8%), apolipoprotein B (5.4 ± 1.9%) but had no effect on serum lipoprotein ratios at week 3 compared to control. However, protein thiol groups were significantly lower compared to control after treatment with flaxseed, suggesting increased oxidation (Jenkins, 1999).

Another study carried out in female Sprague-Dawley rats fed with 10% flaxseed showed an increased level of serum ALA (3.24%), serum EPA (2.43%), serum DHA (3.1%) and a decrease in serum arachidonic acid (26.2%). These results showed a significant increase in total n-3 fatty acids, and a corresponding decrease in total n-6 fatty acids (Babu, 2000).

Prasad (2001) investigated the effects of isolated SDG (40 mg/kg body wt.) from flaxseed on development of T2DM, in the Zucker Diabetic Fatty (ZDF) rat model. The incidence of disease was 100% in untreated ZDF rats and was 20% in SDG treated ZDF rats by the age of 72 days, suggesting that SDG retarded the development of diabetes. Serum MDA levels increased to a similar extent in both untreated and SDG-treated ZDF rats.



(Modified from Prasad, 2000)

Secoisolariciresinol Diglucoside (SDG)

↓ **Human colonic microflora**

Enterodiol

↓ **Oxidized**

Enterolactone

Figure 14: Conversion of SDG to lignans enterodiol and enterolactone by gut microflora.

2.4 Omega 3 Fatty Acids:

The n-3 polyunsaturated fatty acids are highly unsaturated and have one of the double bonds located at three carbon atoms from the methyl end:

* 18:3 α-linolenic acid

* 20:5 Eicosapentaenoic acid

* 22:5 Docosapentaenoic acid

* 22:6 Docosahexaaenoic acid

α-Linolenic acid (Fig. 15) is not synthesized by humans and its deficiency results in adverse clinical symptoms, including neurological abnormalities and poor growth. Therefore, α-linolenic acid is essential in the diet. α-Linolenic acid is the precursor for synthesis of eicosapentaenoic acid and docosahexaenoic acid which are formed in varying amounts in animal tissues e.g. fatty fish (Simopoulos, 1999).

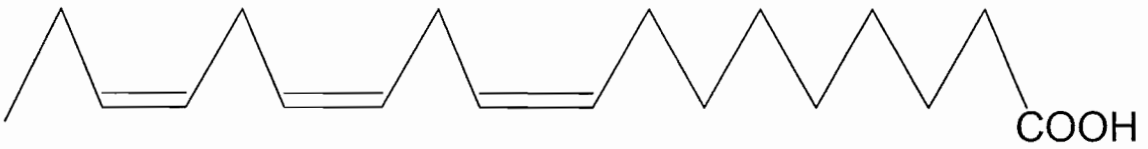


Figure 15: Structure of α-linolenic acid (Modified from Haag, 2003).

When EPA and DHA are ingested, they replace n-6 fatty acids in cell membranes especially those of platelets, erythrocytes, neutrophils, monocytes and liver cells. Higher levels of EPA and DHA leads to:

a. decreased production of prostaglandin E_2 metabolites

b. decreased concentrations of thromboxane A_2, a potent platelet aggregator and vasoconstrictor

c. decreased formation of leukotriene B_4, an inducer of inflammation and a powerful inducer of leukocyte chemotaxis and adherence

d. increased concentrations of thromboxane A_3, a weak platelet aggregator and vasoconstrictor

e. increased concentration of prostacyclin PGI_3 leading to an overall increase in total prostacyclin by increasing PGI_3 without decreasing PGI_2 (both PGI_2 and PGI_3 are active vasodilators and inhibitors of platelet aggregation)

f. increased concentration of leukotriene B_5, a weak inducer of inflammation and chemotactic agent (Simopoulos, 1999).

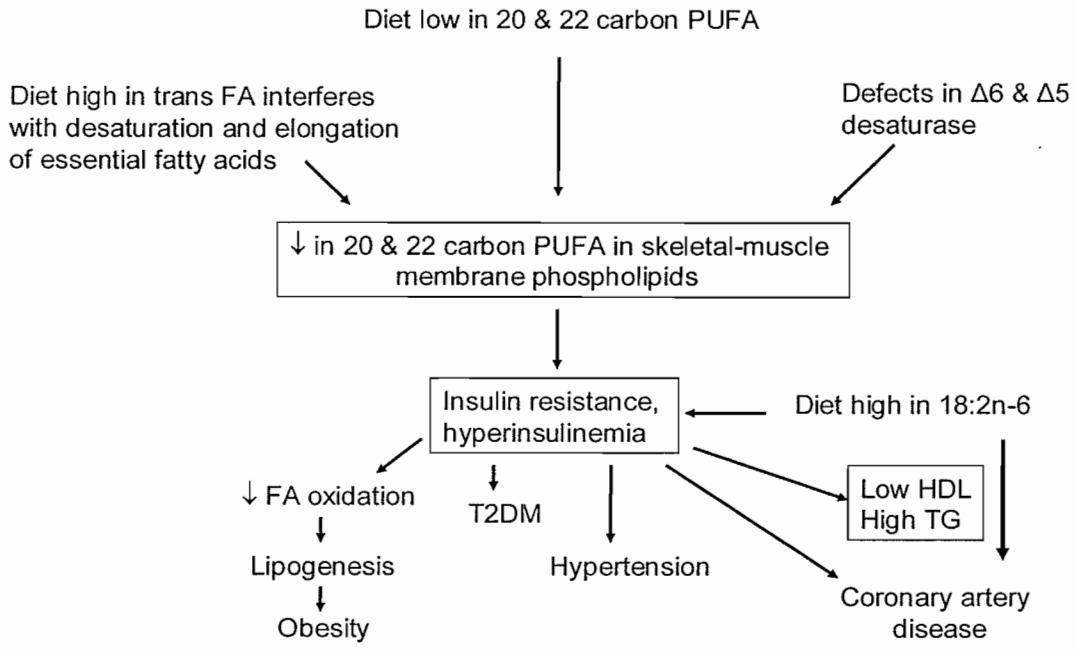
Their beneficial effects have been shown in the prevention and management of coronary heart disease, hypertension, T2DM, renal disease, rheumatoid arthritis, ulcerative colitis, Crohn's disease and chronic obstructive pulmonary disease (Simopoulos, 1999).

Indu & Ghafoorunissa (1992) indicated that increasing dietary ALA increases the EPA concentration in plasma phospholipids after both 3 and 6 wks of intervention to individuals. Dihomo-γ-linolenic acid concentrations were reduced but AA concentrations were not altered. The reduction in the ratio of long chain n-6 PUFAs to long chain n-3 PUFAs was greater after 6 wk than after 3 wk. After ALA supplementation, there was an increase in long chain n-3 PUFA in plasma and platelet phospholipids and a decrease in platelet aggregation but it did not alter serum triacylglycerol concentration.

An increase in 20 and 22 carbon PUFA i.e. AA (arachidonic acid), EPA, and DHA leads to an increase in the membrane fluidity, number of insulin receptors, and

improved insulin action. In humans, the ratio of n-6 to saturated fatty acids in serum phospholipids correlates with insulin sensitivity (Simopoulos, 1999) (Fig. 16).

Connor *et al.,* (1993) convincingly reported that n-3 fatty acid intake in form of fish oil (4.1/1.9 gm EPA/DHA), along with oral therapy for diabetes can lower plasma triacylglycerol concentration with no adverse effect on glycemic control.



(Modified from Simopoulos, 1999)

Figure 16: Hypothetical scheme of the effects of dietary 20 and 22 carbon PUFA in skeletal muscle membrane phospholipids and their relations with insulin resistance, hyperinsulinemia, and chronic disease.

2.5 Metabolic Syndrome:

The metabolic syndrome has become one of the major public health challenges worldwide. The metabolic syndrome is a multifaceted clinical entity resulting from the interaction of genetic, hormonal, and lifestyle factors. Over the past two decades, the number of people diagnosed with the syndrome has steadily increased and is associated with the global epidemic of obesity and diabetes. It is characterized by an aggregation of metabolic risk factors present in an individual. The metabolic syndrome has become a powerful predictor of disease and its diagnosis can be used as a valuable preventive tool (Boehm & Boehm, 2005). The metabolic syndrome is characterized by the clustering of disorders including:

(a) hyperinsulinaemia,

(b) dyslipidaemia,

(c) hypertension, and

(d) excess body weight with central obesity.

Each of these disorders is by itself a risk factor for developing diabetes, and micro-macrovascular complications including coronary heart disease or stroke. The metabolic syndrome has been defined as a specific constellation of risk factors for years and is also known as 'syndrome X', 'the deadly quarter' and 'insulin resistance syndrome' (Boehm & Boehm, 2005).

Kanauchi *et al.,* (2005) reported that pre-diabetes and the metabolic syndrome frequently coexist relatively in lean Japanese and the association seems to link with the abdominal adiposity and insulin resistance. Population-attributable risk estimates associated with metabolic syndrome for cardiovascular disease, coronary heart disease

and T2DM were 34%, 29%, and 62%, respectively in men and 16%, 8%, and 47%, respectively in women (Wilson *et al.,* 2005). They also concluded a rise in the prevalence of the metabolic syndrome over 8 years in a population-based sample that was examined twice.

The metabolic syndrome is a precursor to the development of diabetes. Identifying individuals with the metabolic syndrome is a way to find a large number of patients who are destined to develop diabetes if no intervention is begun at an early stage in their disease (Sorrentino, 2005). It is clear that T2DM is part of a syndrome predisposing affected patients to the development of atherosclerosis. Each factor in its own right is independently associated with increased atherogenic risk; however, the factors may combine to produce a synergistic effect on this risk (Quinn, 2002).

According to the National Cholesterol Education Program (NCEP): Adult Treatment Panel (ATP) III, the metabolic syndrome is defined as 3 or more of the following (Boehm & Boehm, 2005):

Central obesity: Waist circumference >102 cm (male), >88 cm (female)

Hypertriglyceridaemia: triglycerides ≥ 1.7 Mmol/L

Low HDL-C: <1.0 Mmol/L (male), <1.3 Mmol/L (female)

Hypertension: blood pressure ≥ 135/85 mm Hg and/or medication

Fasting plasma glucose: ≥ 6.1 Mmol/L

And according to World Health Organization (WHO) it is defined as diabetes or impaired fasting glycaemia or impaired glucose tolerance or insulin resistance plus 2 or more of the following: (Bernhard *et al.,* 2005).

Obesity: BMI > 30 or waist-to-hip ratio > 0.9 (male) > 0.85 (female)

Dyslipidaemia: triglycerides ≥ 1.7 Mmol/L or HDL-C <0.9 (male) or <1.0 (female) Mmol/L

Hypertension: blood pressure > 140/90 mmHg and/or medication

Microalbuminuria: albumin excretion > 20 µg/min

2.5.1 Mechanisms and low grade inflammation in the metabolic syndrome:

Cellular and molecular mechanisms that link the features of the metabolic syndrome are unknown. Increased adiposity is associated with lipid accumulation in other tissues, including muscle and liver, making it much more difficult to pinpoint the primary sites responsible for initiating metabolic disturbances. Growing evidence links a chronic, subacute inflammatory state to the development of obesity and the metabolic syndrome. Proinflammatory cytokines can cause insulin resistance and anti-inflammatory medications may reverse it, suggesting that inflammation may be directly involved in the pathogenesis. Markers and mediators of inflammation that are biosynthesised in liver like C-reactive protein, plasminogen activator inhibitor-1, fibrinogen and interleukin-6, suggest that 'subacute inflammation' in liver, secondary to steatosis, might be involved in the development of insulin resistance, the metabolic syndrome and subsequently T2DM.

CHAPTER THREE - RATIONALE FOR PRESENT RESEARCH

There is considerable interest in the potential health benefits of oil seeds, such as soy and flaxseed, especially regarding cardiovascular disease, diabetes and cancer. This interest in oil seeds relates to their high content of polyunsaturated fatty acids [particularly ALA], vegetable protein, soluble fibre and flavonoids and related compounds which may possess cholesterol lowering antioxidant and sex hormone agonistic and antagonistic activities (Jenkins, 1999).

Unsaturated fatty acids are generally associated with improved insulin sensitivity. However, there are concerns that consumption of highly unsaturated oils such as flax oil with 55-60% ALA may increase oxidative stress and lead to tissue damage if the capacity of the antioxidant system is compromised. This may occur in these subjects as diabetes and its consequences have been postulated to occur due in part to an inability to cope with over-exposure to reactive oxygen species. Milled flaxseed contains a combination of oil, fibre, lignins and phytochemicals that is expected to reduce oxidative stress. The effect of flax oil itself on oxidative status is less clear. Previous research in hyperlipemic adults suggested that consumption of partially defatted flaxseed may have increased protein oxidation (Jenkins, 1999). Secoisolariciresinol diglucoside from flax has been shown to lead to a reduction in malondialdehyde a breakdown product of lipid peroxidation during the development of diabetes in an animal model and the antioxidant capabilities of SDG and flax meal have been reported (Prasad, 2001 & 1997). Babu (2000) has shown increased Vitamin E in liver of rats fed flax products [one of the major antioxidant in our defense system].

These studies show the potential benefit of flax consumption in a variety of human and animal models confirming the benefit of assessing oxidative status in diabetes.

The present research addresses unanswered questions regarding the consumption of highly unsaturated flax oil and milled flax on antioxidant status in individuals with T2DM. This will provide new data on the efficacy and safety of flax consumption in individuals with T2DM before any recommendations are made.

CHAPTER FOUR - HYPOTHESIS AND OBJECTIVES

Subjects with T2DM were recruited for the present study as intended, but after analysis of their blood (blood glucose, glycated haemoglobin and lipid profile), it was reasonable to conclude that these subjects had Metabolic Syndrome with controlled T2DM managed by diet and exercise. These subjects were not suffering from chronic T2DM, but they were in "pre-diabetes" stage with metabolic syndrome.

HYPOTHESIS:

1. Oxidant status will decrease in those subjects fed milled flaxseed compared to controls as milled flaxseed has antioxidative component SDG.
2. Oxidant status will not increase in those subjects fed flax oil compared to controls as flax oil do not contain antioxidative component SDG.

Oxidant status can be described as sum of oxidant stress and antioxidant ability.

OBJECTIVES:

Assess oxidative status in subjects (n=35) with metabolic syndrome who consumed flax products (flaxseed oil, milled flax or control) for 12 weeks.

1. Measure the ability to resist oxidative stress in plasma.
2. Measure endogenous antioxidant enzyme SOD and CAT activity in erythrocytes.
3. Measure Vitamin A (retinol), Vitamin C and Vitamin E in plasma.
4. Measure hydroperoxide levels in plasma.
5. Measure F_2 isoprostanes in urine.

CHAPTER FIVE - METHODS

5.1 Subjects and study design:

A total of 35 subjects (17 males and 18 females; age range 33-66) having controlled T2DM were studied. These subjects had been diagnosed with T2DM between 1 month and 11 years previously, when they were enrolled in the study. Subjects were divided into three groups receiving food products containing (a) milled flaxseed (milled flax group, n=13) 7.6 gm of ALA per day for 6 days per week, (b) flaxseed oil (flax oil group, n=12) 7.6 gm of ALA per day for 6 days per week and (c) products without flaxseed (control group, n=10) for 12 weeks. At baseline (week 0) all the three groups were same i.e. no flax supplementation was given. The dose of flaxseed for this study was decided on the basis of the amount used in the previous studies and their results (Prasad 2000 & 2001, Babu *et al.,* 2000, Wiesenfeld *et al.,* 2003, Cunnane *et al.,* 1993 & 1995, Jenkins *et al.,* 1999, Lee *et al.,* 2003). Background information (Appendix 9) taken on these subjects revealed that the majority of them had complications such as hypercholesterolemia, hypertension, stroke, arthritis and few subjects had undergone recent surgery prior to the study.

Fasting blood samples were collected into tubes containing EDTA were centrifuged to separate plasma and erythrocytes for biochemical analysis. Blood samples and 24-hour urine samples were collected at 4 time points: baseline (0), 4, 8, and 12 weeks.

5.2 Measuring hydroperoxides in plasma by the FOX2 assay:

Hydroperoxides are the initial stable products formed during peroxidation of unsaturated lipids such as fatty acids or cholesterol. Oxidation of unsaturated lipids involves an allylic hydrogen abstraction, insertion of molecular oxygen, and subsequent reduction of the resultant hydroperoxyl radicals to hydroperoxides (Nourooz-Zadeh, 1994).

The FOX assay is based on the oxidation of ferrous to ferric ions by ROOHs under acidic conditions. The dye xylenol orange [o-cresolsulfonphthalein-3,3-bis (methliminodiacetic acid sodium salt)] complexes with an equal molar concentration of ferric ion to produce a color with an apparent extinction coefficient at 560 nm of 1.5 x 10^4 M^{-1} cm^{-1}. Undesirable chain oxidation is prevented by inclusion of the lipid soluble chain breaking antioxidant butylated hydroxytoluene (BHT) which repairs the alkyl radicals produced by the reaction of alkoxyl with unsaturated lipids (Nourooz-Zadeh *et al.,* 1994).

$$ROOH + Fe^{+2} \rightarrow Fe^{+3} + RO^{\cdot} + {}^{-}OH$$

$$RO^{\cdot} + RH \rightarrow ROH + R^{\cdot}$$

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$$

$$ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}$$

$$R^{\cdot} + BHT \rightarrow RH + BH^{\cdot}$$

The FOX 2 assay uses conjugation with triphenylphosphine (TPP) and has been implemented for the measurement of plasma ROOHs (Nourooz *et al.,* 1994, 1995 & 1997). TPP reduces ROOHs to their corresponding alcohols while itself being converted

to triphenylphosphine oxide. Other advantages of the FOX 2 assay over existing techniques includes: (a) the kinetics of the reaction are independent of the chemical structure of ROOHs, and (b) no extraction step is normally needed for analysis of liposomes and lipoprotein suspensions because of the use of 90% methanol/25 Mmol H_2SO_4 which denatures proteins sufficiently for access of the ferrous ions to available ROOHs. Reported hydroperoxide plasma values of healthy individuals are in the range of 0.22 – 7.8 μM (Nourooz *et al.,* 1994). Appendix 7 describes the FOX 2 assay.

5.3 Measuring total antioxidant property by using the FRAP assay:

The ferric reducing/antioxidant power (FRAP) assay is a recently developed, direct test of "total antioxidant property". Other tests of total antioxidant power used to date are indirect methods that measure the ability of antioxidants in the sample to inhibit the oxidative effects of reactive species purposefully generated in the reaction mixture. In inhibition assays, antioxidant action induces a lag phase; exhaustion of antioxidant power is denoted by a change in signal such as rate of oxygen utilization fluorescence, or chemiluminescence. Measurement of these signals requires specialized equipment, and such tests can be time consuming, technically demanding and may lack sensitivity (Benzie, 1999).

In contrast to other tests of total antioxidant power, the FRAP assay is simple, speedy, inexpensive and robust. The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method; the FRAP does not use a lag phase type of measurement. In the FRAP assay, sample pre-treatment is not required, stoichiometric factors are constant, linearity is maintained over a wide range, reproducibility is

excellent, and sensitivity is high. FRAP does not require highly specialized equipment or skills, or critical control of timing and reaction conditions. FRAP values of fresh, fasting plasma from healthy adults range from 638 -1634 μM (Benzie, 1999).

Concept of the FRAP assay:

A biological antioxidant has been defined as "any substance that when present at low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substrate" (Benzie, 1999). In simple terms, electron-donating antioxidants can be described as reductants, and inactiviation of oxidants by reductants can be described as redox reactions in which one reactive species is reduced while another is oxidized. In this context therefore, "total antioxidant power may be referred to analogously as total reducing power" (Benzie, 1999). The assay procedure is described in Appendix 1.

5.4 Measuring Vitamin C by Omaye method:

Ascorbic acid is oxidized by copper to form dehydroascorbic acid and diketogulonic acid. These products are treated with 2,4-dinitrophenylhydrazine to form the derivative bis-2,4-dinitrophenylhydrazone. This compound, in strong sulfuric acid, under goes a rearrangement to form a product with an absorption band that is measured at 520nm. The reaction take place in presence of thiourea medium which helps to prevent interference from non-ascorbic acid chromgenes. Normal values of ascorbic acid in human plasma are reported to be between 0.8 -1.5 mg/dl (Omaye *et al.,* 1979). See Appendix 2 for the assay procedure.

5.5 Measuring Vitamin E and Vitamin A in plasma by HPLC:

Fat soluble Vitamins in the samples were extracted in organic solvent and then concentrated. Extracted Vitamins were determined by HPLC on C18 column. All work was done in subdued light to avoid oxidation. Solvent evaporation was done under nitrogen. Normal plasma tocopherol values have reported to be in the range of 5-16 μg/ml (Farrell *et al.,* 1978 & Herbeth *et al.,* 1986) and those of Vitamin A are observed between 0.55 - 0.97 μg/ml (Herbeth *et al.,* 1986). The assay procedures are described in Appendix 3.

5.6 Measuring antioxidant enzymes:

5.6.1 Measuring catalase activity by Aebi's method:

Catalytic activity is present in nearly all animal cells and organs and in aerobic microorganisms. Catalase exerts a dual function: (a) decomposition of H_2O_2 to give H_2O and O_2 and (b) oxidation of H donors.

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

$$ROOH + AH_2 \longrightarrow H_2O + ROH + A$$

The predominating reaction depends on the concentration of H donor and the steady-state concentration or rate of production of H_2O_2 in the system. In both cases the active catalase-H_2O_2 I complex is formed first. The decomposition of H_2O_2, in which a second molecule of H_2O_2 serves as H donor for complex I, proceeds exceedingly rapidly whereas peroxidative reactions proceed relatively slowly.

In the ultraviolet range, H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the

decrease in absorbance at 240 nm. The difference in absorbance per unit time is a measure of the catalase activity. One unit is defined as millimole H_2O_2 consumed/min/mg protein and specific activity is expressed as U/mg protein. Erythrocyte catalase activity in normal healthy adults were reported to be in range of 990-1300 U/mg protein (Marklund *et al.,* 1982) and for normal healthy infants it ranges from 76-94 U/mg protein (Friel *et al.,* 2004). Appendix 4 describes the assay procedure.

5.6.2 Measuring superoxide dismutase activity by Spitz's method:

The nitroblue tetrazolium (NBT) bathocuproine sulphide (BCS) SOD assay examines the catalytic conversion of xanthine to uric acid and O_2 by xanthine oxidase which provides an initial constant flux of superoxide in a phosphate buffered solution at room temperature. The rate of NBT reduction to blue formazan by the xanthine oxidase reaction is monitored spectrophotometrically at 560 nm. When increasing concentrations of pure SOD enzyme or protein homogenate containing SOD activity are added to the reaction, the rate of NBT reduction by xanthine/xanthine oxidase is progressively inhibited until maximum inhibition is reached for the NBT-BCS assay. Catalase is added to the reaction to remove hydroperoxide. Reported erythrocyte SOD activity in normal healthy women was between 99-282 U/mg protein (Carmeli, 2004). Appendix 6 describes the assay procedure.

5.7 Estimation of protein in erythroctyes:

The procedure described here is based on Peterson's modification of the micro Lowry method and utilizes sodium dodecylsulfate, included in the Lowry Reagent, to facilitate the dissolution of relatively insoluble lipoproteins. The procedure is based on

two chemical reactions, (a) the biuret reaction, in which the alkaline cupric tartrate reagent complexes with the peptide bonds of the protein, (b) the reaction of the Folin and Ciocalteu's phenol reagent, which yields a purple color which is read at 750 nm. The protein concentration is determined from a calibration curve. The method is described in Appendix 5.

5.8 Measuring 8-isoprostanes in urine:

This photometric assay has been validated to measure 8-isoprostane in wide range of samples including urine and plasma. This assay is based on competition between 8-Isoprostane and an 8-Isoprostane-acetylcholinesterase (AChE) conjugate (8-Isoprostane tracer) for a limited number of 8-Isoprostane specific rabbit antiserum binding sites. Because the concentration of 8-isoprostane tracer is held constant while the concentration of 8-isoprostane varies, the amount of 8-isoprostane that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of 8-isoprostane in the sample well. This rabbit antiserum 8-isoprostane complex binds to the rabbit IgG mouse monoclonal antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent is added to the well. The product of this enzymatic reaction has a distinct yellow color (412 nm) which is proportional to the amount of 8-isoprostane tracer bound to the well, which is inversely proportional to the amount of free 8-isoprostane present in the well during incubation. The assay procedure is described in Appendix 8.

CHAPTER SIX – STATISTICS

Data were analyzed for statistical significance by two-way analysis of variance (ANOVA) with repeated measures, with time and group as factors. Time, group effects and time×group interactions were considered significant at $p<0.05$. When a main effect was statistically significant, Tukey's test was done for the comparison of means and $p<0.05$ was considered as significant. When the time×group interaction was significant, pre-planned estimates were used and significance was adjusted to $p<0.004$ to be significant at 5% level. All values for biochemical parameters are expressed as mean ± SE (standard error).

The normality of the data for each variable was checked by examining Pearson's residual quantile plots. Potential outliers, which were extreme outside ± 3SD (standard devitation), were removed for the variables CAT and Vitamin A and residual plots were ran again to check for the normality.

CHAPTER SEVEN – RESULTS

7.1 Antioxidant Vitamins in plasma:

7.1.1 Vitamin E:

Plasma Vitamin E levels were unaffected in the milled flax and flax oil groups when compared to the control group over the study period (Fig. 17 and Appendix-Table 5). None of the treatments showed variation in the plasma Vitamin E levels of these subjects.

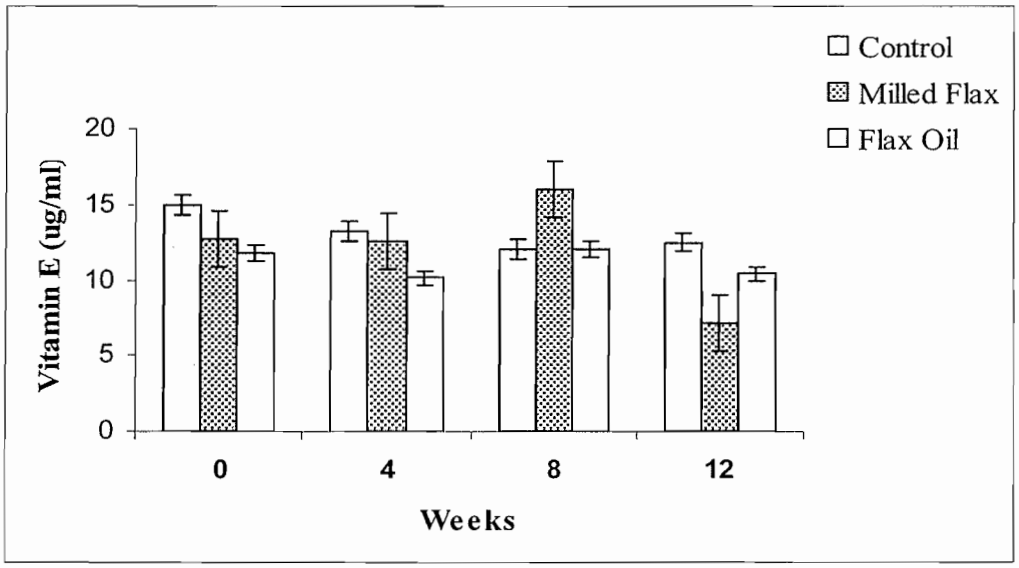


Figure 17: Plasma Vitamin E (μg/ml) levels of the groups over the period of 12 weeks. Values are expressed as Mean ± SE, control (n=10), milled flax (n=13) and flax oil (n=12).

The Vitamin E levels for all the groups are within the normal range of healthy adults. Normal plasma tocopherol values have reported to be in range of 5 -16 μg/ml (Farrell *et al.,* 1978 & Herbeth *et al.,* 1986).

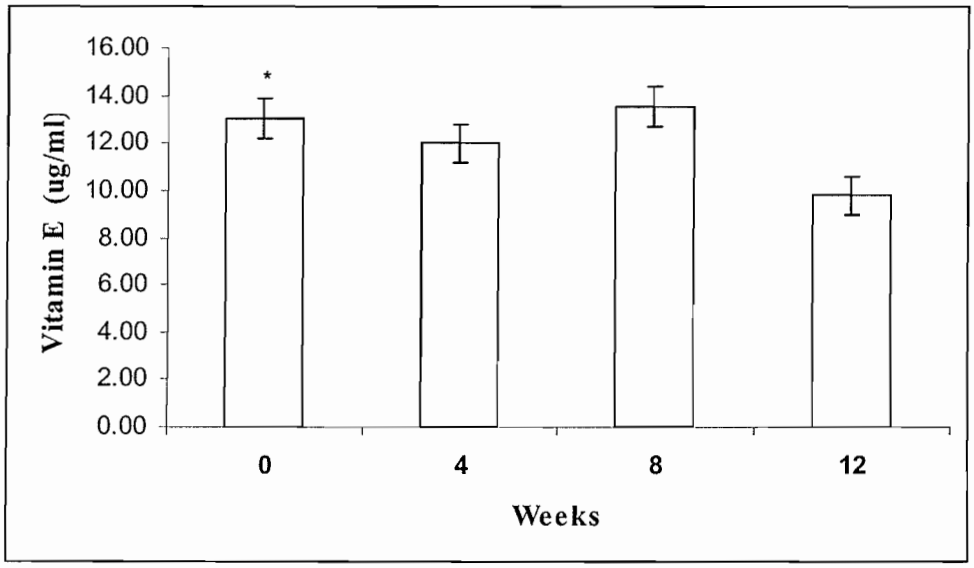


Figure 18: Plasma Vitamin E level of all the groups at each time points. A significant time effect (p=0.001) was seen. *Mean value at week 0 was significantly different from those at other weeks (Tukey's test).

Plasma Vitamin E levels were different at 0 week when compared to other time points (Fig. 18).

7.1.2 Vitamin C:

Plasma Vitamin C levels in the flax oil group were found to be significantly different from the milled flax group at week 4 (Fig. 19). Otherwise, neither of the treatments showed any effect on the plasma Vitamin C levels of these subjects at other time points. The levels of Vitamin C of the milled flax group at 0 and 12 weeks fall within the normal range of healthy adults, other values tend to be relatively low (Appendix-Table 5). Normal values of ascorbic acid in plasma are reported between 0.8-1.5 mg/dl (Omaye *et al.*, 1979).

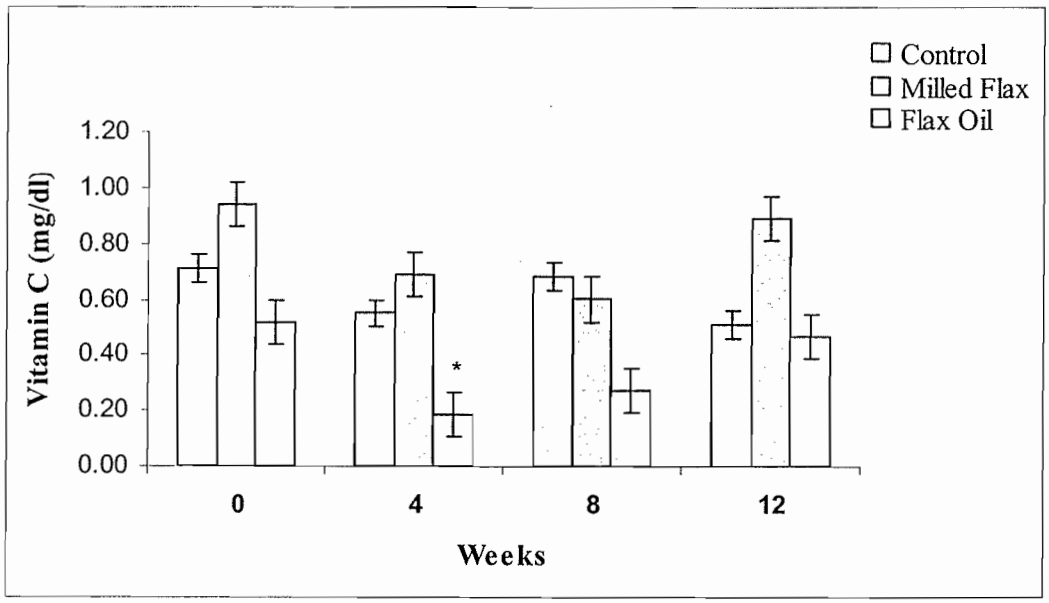


Figure 19: Plasma Vitamin C (mg/dl) levels of the groups over the period of 12 weeks. Values are expressed as Mean ± SE, control (n=10), milled flax (n=13) and flax oil (n=12). A significant time effect (p=0.0002), group effect (p=0.01) as well as group & time interaction (p=0.03) was seen. *Mean of the flax oil group is different (p<0.004) from the milled flax group at week 4.

7.1.3 Vitamin A

No statistical significant difference was seen in plasma Vitamin A levels among the study groups over the period of 12 weeks (Fig. 20 and Appendix-Table 5). Normal plasma Vitamin A values observed in literature are between 0.55-0.97 μg/ml (Herbeth *et al.,* 1986). Plasma Vitamin A levels of these subjects tend to be higher than normal individuals.

There was a significant time effect where, Vitamin A levels at week 4 were different from week 8 and levels at week 8 were different from week 12 (Fig. 21).

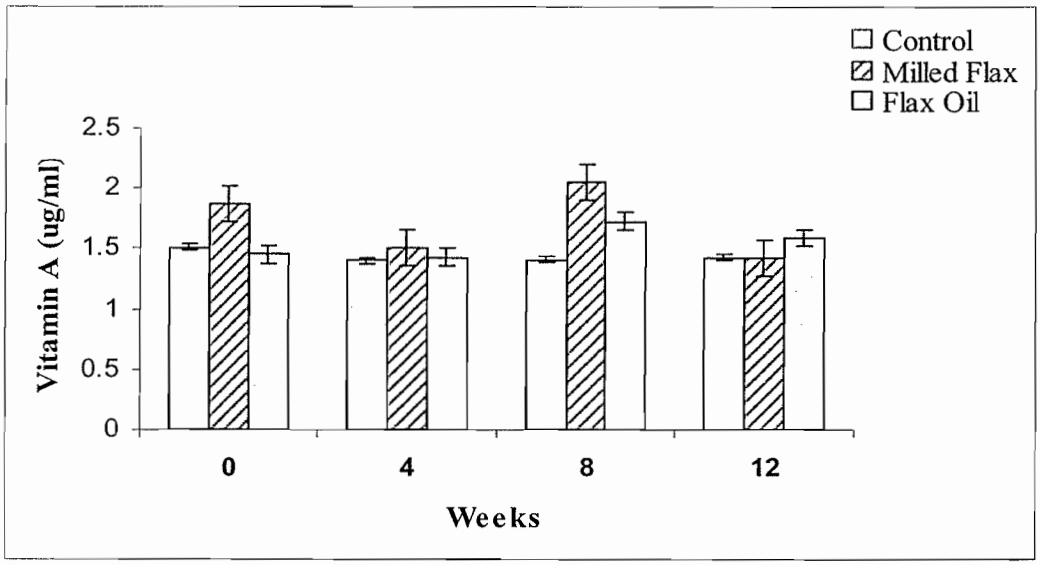


Figure 20: Plasma Vitamin A (µg/ml) levels of the groups over the period of 12 weeks. Values are expressed as Mean ± SE, control (n=10), milled flax (n=13) and flax oil (n=12).

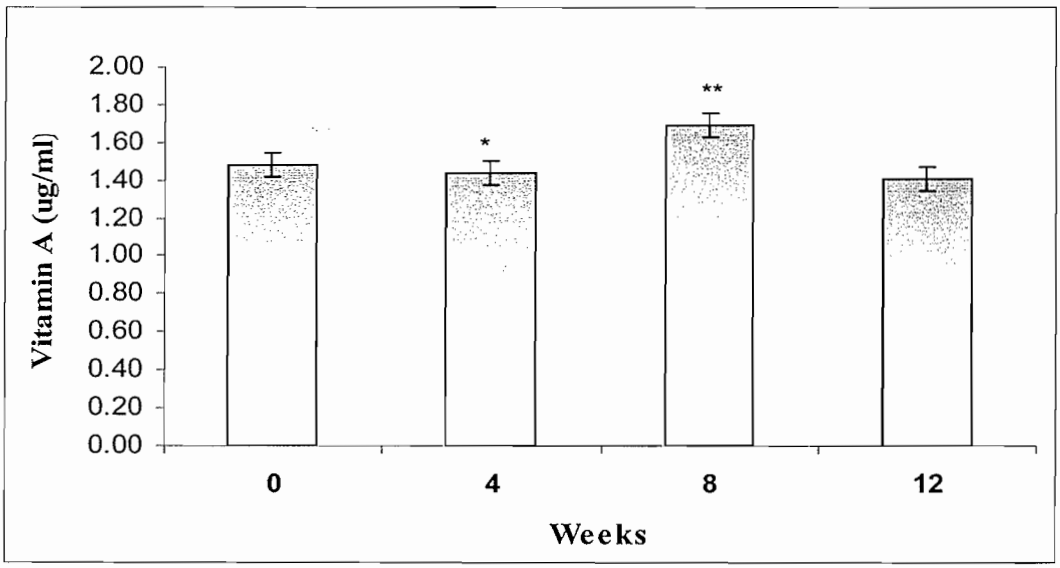


Figure 21: Plasma Vitamin A level at each time points. A significant time effect (p=0.01) was seen. * Mean of the groups at week 4 is different from week 8. **Mean of the groups at week 8 is different from week 12.

In conclusion, some variations seen in Vitamins A, E and C at different weeks could be due to the difference among the individuals or due to their dietary pattern. However, it is evident that time has an effect on these parameters irrespective of the treatment.

7.2. Enzymatic antioxidant activity in erythrocytes:

7.2.1 Catalase

At week 0, erythrocyte catalase activity was significantly higher in the milled flax group when compared to the flax oil and the control groups (Fig. 22). In addition, at week 4 the erythrocyte catalase activity in the flax oil group was statistically higher than the milled flax group. No change in the erythrocyte catalase activity was seen among the groups at 8 and 12 weeks (Appendix-Table 5).

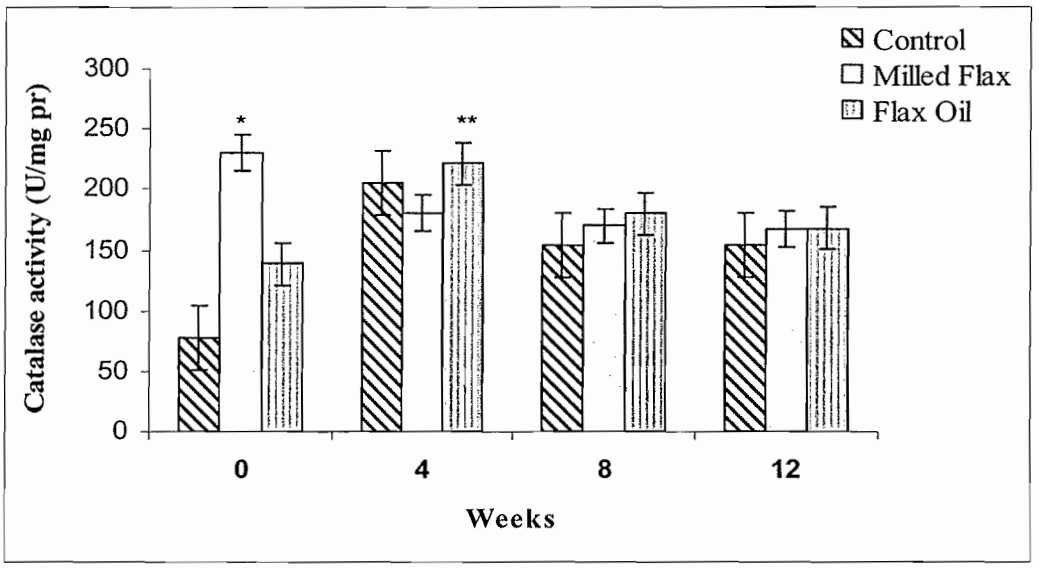


Figure 22: Erythrocyte catalase activity (U/mg protein) of the groups over the period of 12 weeks. Values are expressed as Mean ± SE, control (n=10), milled flax (n=13) and flax oil (n=12). A significant time effect (p=0.02), group effect (p=0.01) as well as group and time interaction (p<0.0001) was seen. *Mean of the milled flax group is different (p<0.004) from the other groups at week 0. **Mean of the flax oil group is different (p<0.004) from the milled flax group at week 4.

Erythrocyte catalase activity in normal healthy adults ranges from 990-1300 U/mg protein (Marklund *et al.,* 1982) and in normal healthy infants ranges from 76-94 U/mg protein (Friel *et al.,* 2004).

7.2.2 Superoxide Dismutase

Only at week 0, erythrocyte SOD activity was found to be higher in the milled flax group compared to the other study groups (Fig. 23 and Appendix-Table 5). Normal Cu-Zn SOD and the SOD activity in the erythrocytes among healthy adults have been reported to range from 61-67 U/mg protein (Marklund *et al.,* 1982) and 99-282 U/mg protein (Carmeli *et al.,* 2004).

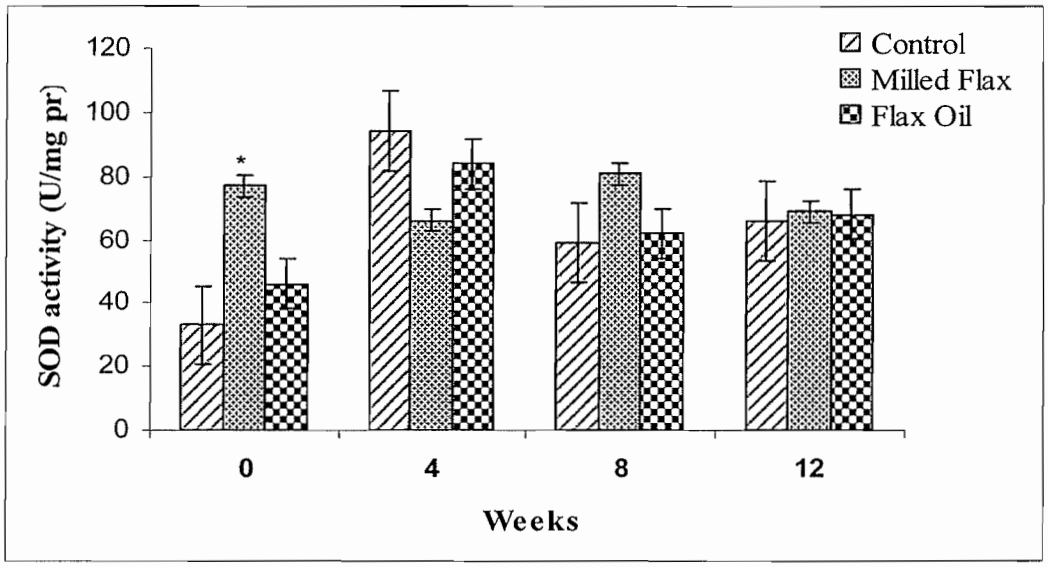


Figure 23: Erythrocyte superoxide dismutase activity (U/mg protein) of the groups over the period of 12 weeks. Values are expressed as Mean ± SE, control (n=10), milled flax (n=13) and flax oil (n=12). A significant time effect (p=0.001) and group & time interaction (p<0.007) was seen. *Mean of the milled flax group is different (p<0.004) from other two groups.

The SOD and CAT activity of these subjects is slightly lower than the normal, but a parallel trend was observed over period of 12 weeks. This could be due to the presence of number of factors like metabolic syndrome or inadequacy of minerals like copper, zinc, selenium or individual variation. However, it is interesting to observe that both the enzymes are different only at week 0 in the milled flax group.

7.3 Total antioxidant property in plasma:

Total antioxidant property in plasma was unchanged over the period of 12 weeks in the three groups (Fig. 24). Consumption of the flax oil or the milled flax did not show any effect on the total antioxidant property of these subjects, when the control, milled flax and flax oil groups were compared. FRAP values of the milled flax group seems to be lower than the other study groups but no statistical difference was observed. The FRAP levels for all the groups were found to be within the normal plasma range of healthy adults (Appendix-Table 5). FRAP value of fresh, fasting plasma from healthy adults ranges from 638-1634 μM (Benzie, 1999).

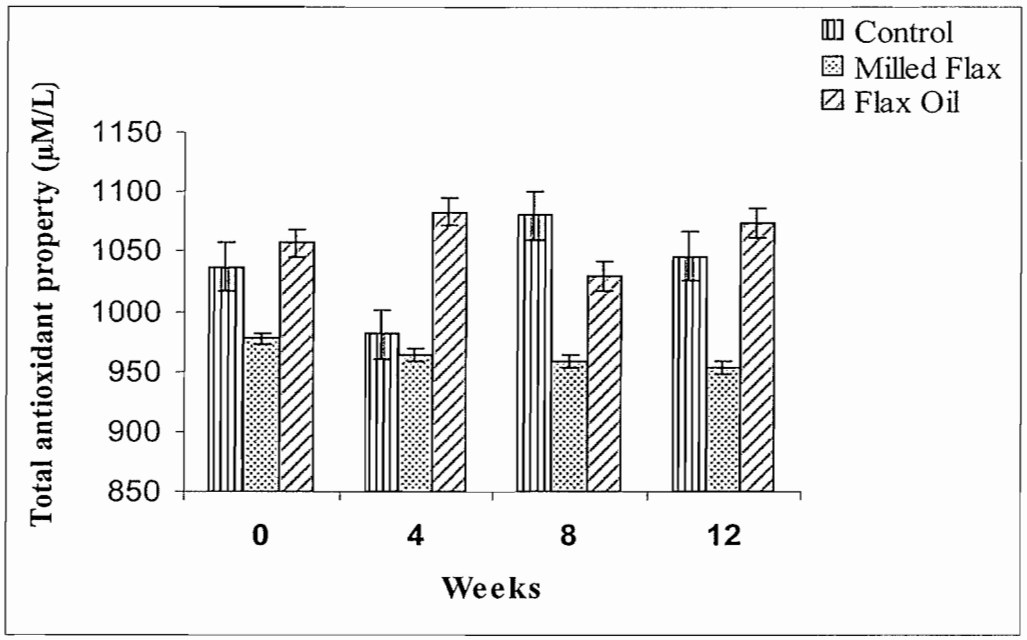


Figure 24: Plasma total antioxidant property (FRAP) (μM/L) of the groups over the period of 12 weeks. Values are expressed as Mean ± SE, control (n=10), milled flax (n=13) and flax oil (n=12).

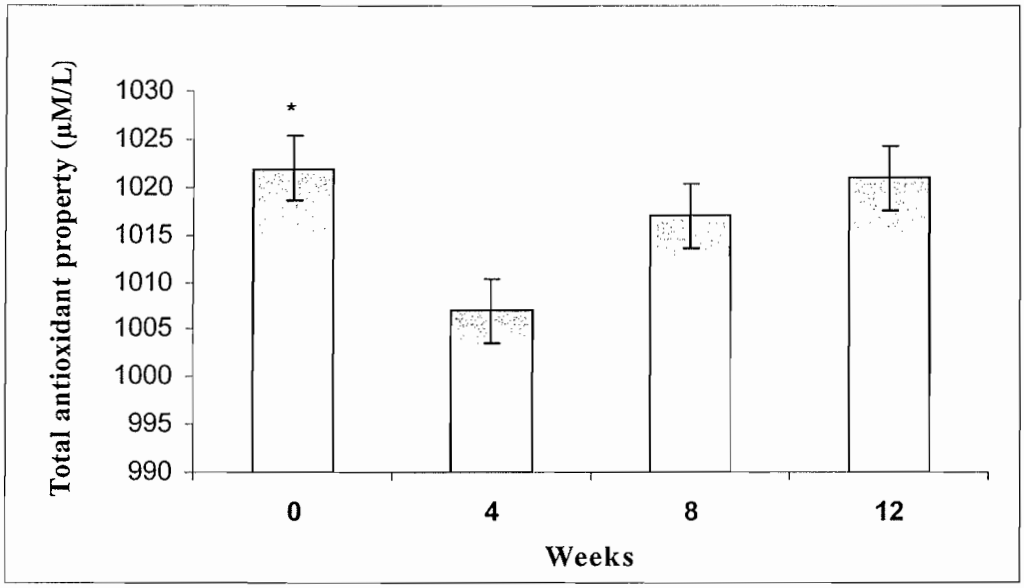


Figure 25: Plasma total antioxidant property at each time point. A significant time effect (p=0.001) was seen. *Mean at week 0 is different from the other time points (Tukey's test).

The total antioxidant property at week 0 was significantly (Fig. 25) different from 4, 8, and 12 weeks.

7.4. Hydroperoxide levels in plasma:

No difference in plasma hydroperoxide levels was seen among the three groups over the study period (Fig. 26). At week 0, the plasma levels of hydroperoxides in all the groups are just slightly higher then the levels at 4, 8, and 12 weeks but they are not statistically different. The plasma hydroperoxide levels of all the groups were within the normal range of hydroperoxides in healthy adults (Appendix-Table 6). Plasma of healthy individuals contains hydroperoxides in the range of 0.22 – 7.8 µM (Nourooz *et al.,* 1994).

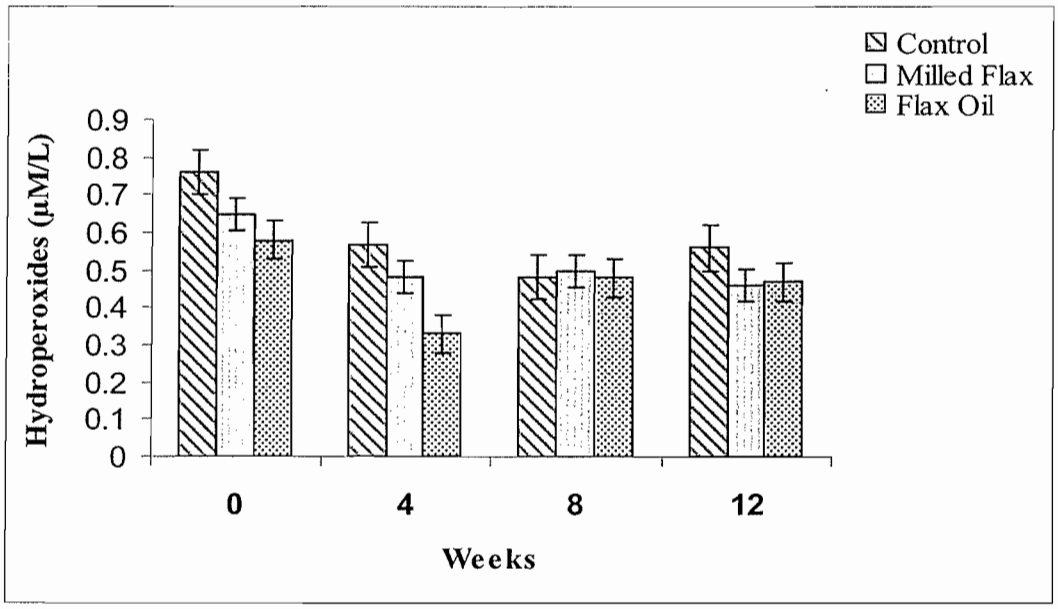


Figure 26: Plasma hydroperoxide (µM/L) levels of the groups over the period of 12 weeks. Values are expressed as Mean ± SE, control (n=10), milled flax (n=13) and flax oil (n=12).

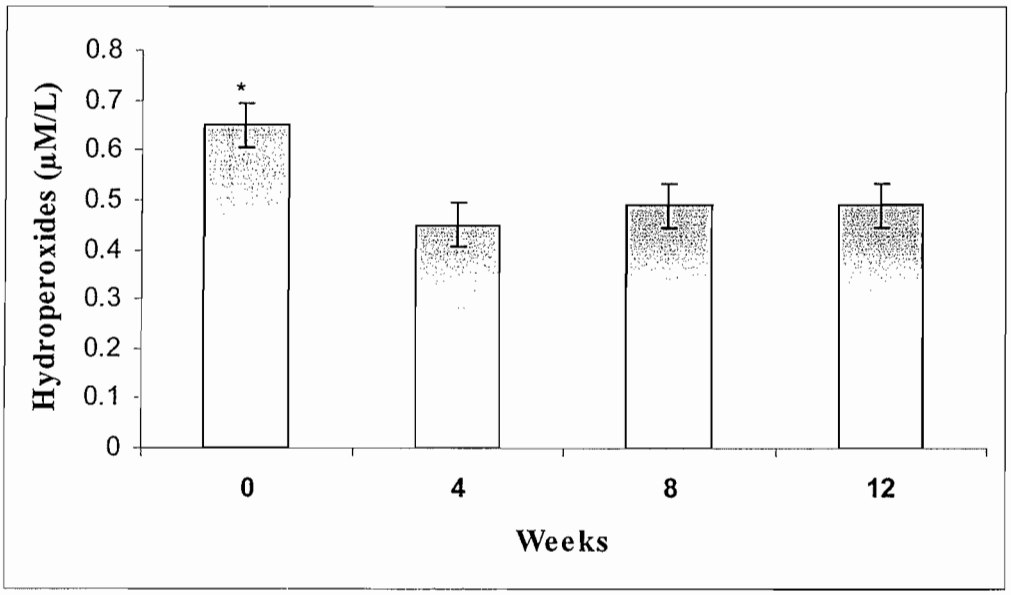


Figure 27: Plasma hydroperoxide level at each time points. A significant time effect (p=0.001) was seen. *Mean at week 0 is different from the other time point (Tukey's test).

Hydroperoxide level at the baseline (Fig. 27) was significantly higher than the other time points.

7.5. Urinary Isoprostanes:

Isoprostane levels of the milled flax and flax oil group were significantly lower than the control group at all time point (Fig. 28 and Appendix-Table 6). No difference was seen in the isoprostane levels when the milled flax and flax oil groups were compared. This could be due to the within and between variations of the groups.

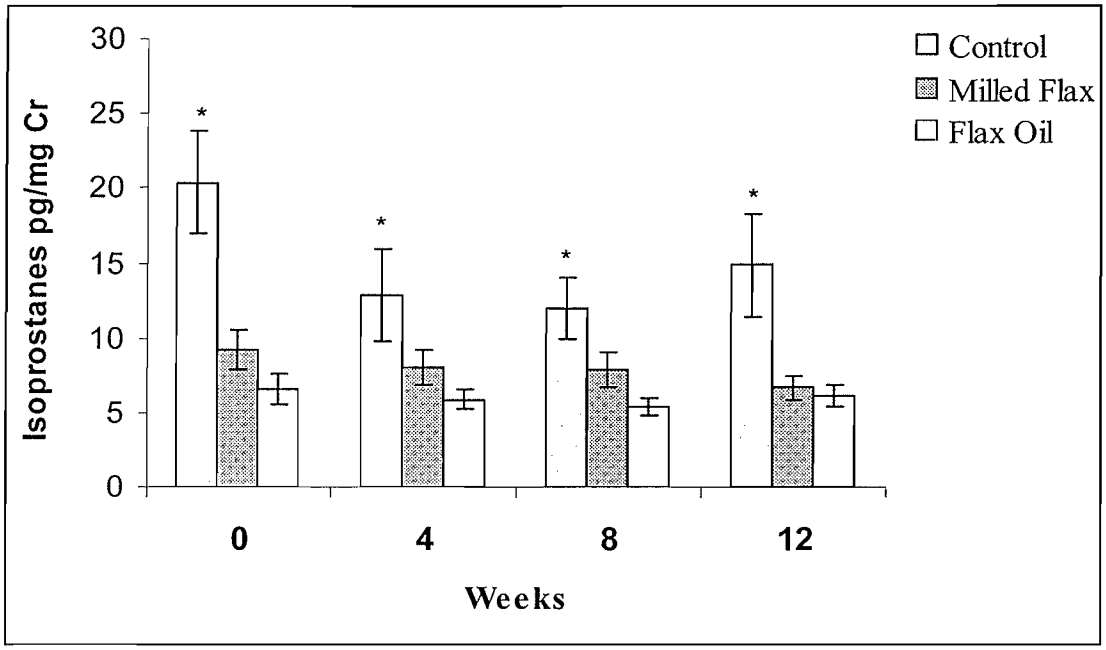


Figure 28: Urinary isoprostane (pg/mg creatinine) levels of the groups over the period of 12 weeks. Values are expressed as Mean ± SE, control (n=10), milled flax (n=13) and flax oil (n=12). *Mean of the control group is higher ($p<0.004$) from the means of the treatment groups at all time point.

In normal human varied levels of urinary isoprostane are reported to be around 180 – 2600 pg/mg creatinine, 41 – 433 pg/mg creatinine and 460 pg/mg creatinine (Il'yasova *et al.,* 2005, Davi *et al.,* 1999 and Morrow & Roberts, 2002). Urinary levels of isoprostanes of all the three groups were relatively lower over the period of 12 weeks when compared to the normal range.

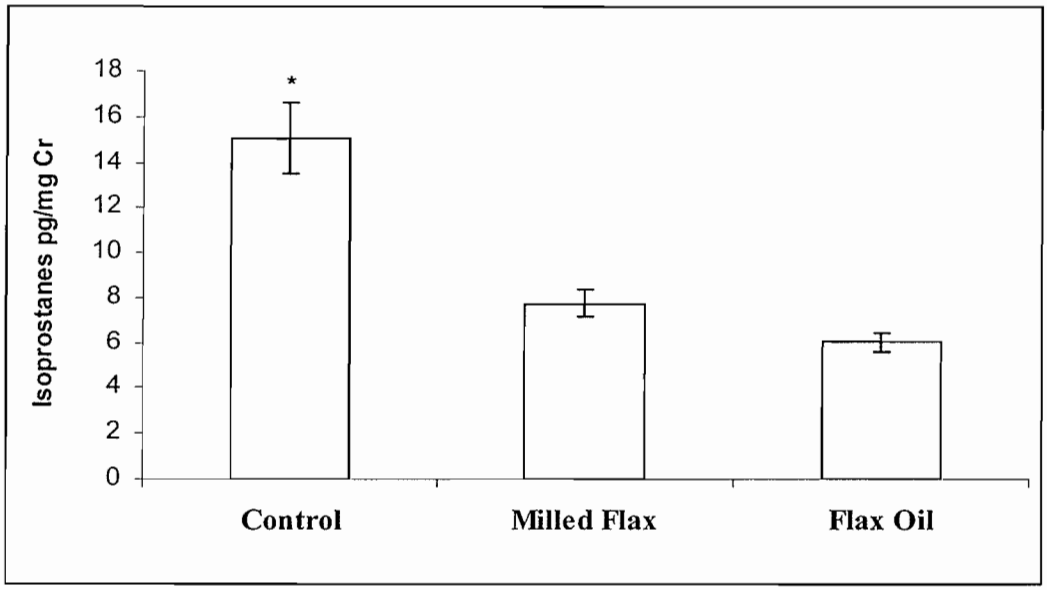


Figure 29: Urinary isoprostane (pg/mg creatinine) level of the treatment groups. A significant main group effect (p=0.0002) was seen. *Mean of the control group was higher from the milled flax and flax oil groups (Tukey's test).

Urinary isoprostanes were significantly higher in the control group (Fig. 29) when compared to the treatment groups. Levels at week 0 were significantly different from those at 4 and 8 weeks (Fig. 30).

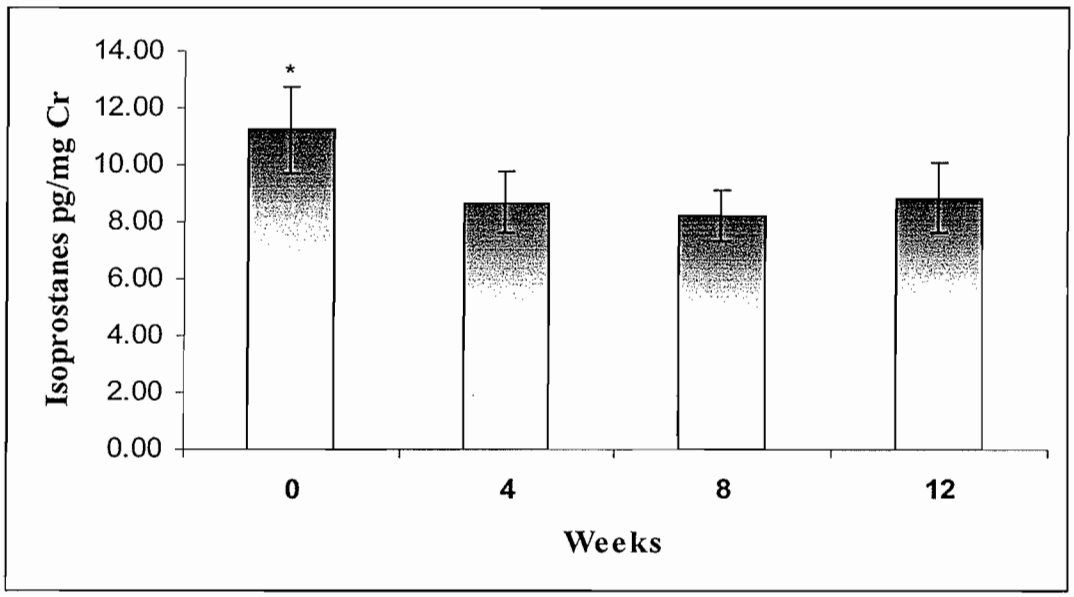


Figure 30: Urinary isoprostane (pg/mg creatinine) at each time point. A significant main time effect was seen (p=0.01). *Mean of 0 week is different from 4 and 8 weeks (Tukey's test).

Overall, the results showed no effect of the flaxseed consumption on the biochemical parameters measured. However, large within and between variability were seen in the individuals and among the groups over the duration of the study. This could be one of the reason that some level of significance is seen in the groups for a few of the parameters at different time points.

CHAPTER EIGHT – DISCUSSION

Oxidative stress results when the rate of oxidant production exceeds the rate of oxidant scavenging. Reactive oxygen species are increased by hyperglycemia (King & Loeken, 2004). Oxidative stress may be important in diabetes, not just because of its role in the development of complications, but because persistent hyperglycemia secondary to insulin resistance, may induce oxidative stress and contribute to beta cell destruction in T2DM (King & Loeken, 2004). Hyperglycaemia can degrade antioxidant enzyme defenses, thereby allowing reactive oxygen species to damage other enzymes and structural proteins (West, 2000). Sundaram *et al.,* (1996) reported that plasma and erythrocyte TBARS are significantly elevated in T2DM and appear to be associated with the multiplicity of complications and duration of diabetes mellitus.

Cell defenses against toxic oxygen include enzymatic antioxidants as well as non-enzymatic antioxidants. Flaxseed is a rich source of alpha linolenic acid (59%) and the plant lignan secoisolariciresinol diglycoside (SDG). SDG in flaxseed has been shown to be metabolized in the body by colonic microflora to enterodiol and enterolactone (Prasad, 2001) in both *in vivo* fermentation and *in vitro* studies (Kitts *et al.,* 1999). Also, it has been reported that enterodiol and enterolactone have three times greater antioxidant property than SDG. The lower antioxidant activity of SDG compared to enterodiol and enterolactone may involve the lack of positioning of phenolic hydroxyl groups toward free radicals, thus preventing the ring-structure hydroxyl groups from acting as hydrogen donors and free radical scavengers (Kitts *et al.,* 1999).

To assess the effects of flaxseed on diabetic progress, we studied three groups of subjects: (a) Control (b) Milled flaxseed and (c) Flax oil. Subjects were both male and female between 33 and 66 years of age and having an average fasting glucose of 7.25 Mmol/L and BMI of 33. A majority of these subjects took medications to control other conditions like hypertension, hypercholesterolemia, and hypertriglyceridaemia (1.85 Mmol/L). When they were enrolled in the study these subjects had been diagnosed with T2DM between 1 month and 11 years previously. They controlled their diabetes by diet and exercise and monitored blood glucose levels on a regular basis. As a result, when their blood glucose was analyzed, the levels were found to be in the pre-diabetic stage. In addition, their glycated haemoglobin (6.9) did not support that they had diabetes during the study. It was concluded that these subjects did not have T2DM, but they have the metabolic syndrome. Subjects with T2DM managed by oral agents or insulin, taking flaxseed or omega 3 fatty acids in any form prior to the study were excluded.

We studied the total antioxidant capacity, including antioxidant enzyme activity in erythrocytes and water-lipid soluble antioxidant Vitamins in plasma. In the same samples, we studied the extent of ROOH in plasma and 8-isoprostane in urine, in order to verify the possible correlation between the cellular enzymatic antioxidant capacity and the degree of membrane lipid peroxidation. Plasma protein carbonyls in some of these subjects were analyzed at week 0 and 12 by another lab at Health Canada in Ottawa.

In this study, plasma Vitamin E levels were unaffected by the dietary treatments, however, a significant time effect was seen. Similar, results where plasma Vitamin E levels were not affected by the flaxseed consumption are reported by Cunnane *et al.,* (1994) in healthy young controls (50 gm flaxseed/day) and by Babu *et al.,* (2000) in

weanling female Sprague-Dawley rats. Wiesenfeld *et al.,* (2000) reported similar findings of no change in serum Vitamin E levels, but showed a significant decrease in liver and heart Vitamin E in rats fed 40% dietary flaxseed. On the contrary, Babu *et al.,* (2000) reported an increase in Vitamin E storage in liver by moderate consumption of dietary ground flaxseed and defatted flaxseed meal, which was attributed to the antioxidant effects of the lignans present in the flaxseed. It is also speculated that lignans protect the tocopherols *in vivo* or increase the binding activity of the tocopherol-binding protein (Babu *et al.,* 2000). In conclusion, plasma Vitamin E levels were not altered in any of the groups by the consumption of flax oil or milled flax.

Among the studies carried out so far with flaxseed consumption in diabetics and normal individuals, very few studies have reported its effect on the antioxidants Vitamin C and A. Our results did not show any significant difference in plasma levels of Vitamin C and A in any of the treatment groups, lower Vitamin C levels were seen in the flax oil group compared to the milled flax group at week 4. Vitamin A levels were higher than the normal range whereas Vitamin C levels inclined to be lower than the normal range. A significant main time effect was seen for Vitamin A, mean of the groups at week 4 is different from week 8 and mean of the groups at week 8 is different from week 12. A significant main time effect was also seen with Vitamin E, mean at week 0 is different from other time points. This could be due to the individual metabolic variation, or their dietary intake or compensatory mechanism of the body or due to day-to-day analytical variation. Wiesenfeld *et al.,* (2000) reported a U-shaped dose response for Vitamin A levels in liver, where animals fed 13% defatted flax meal showed stimulatory effects compared to animals fed 26% and 40% defatted flax meal. Gallan *et al.,* (2003) reported

that the values of total antioxidant status, Vitamin A or E were not decreased in well-controlled young type 1 diabetes patients and no significant differences were noted between the subgroups of patients classified by subclinical complications. Neither of the antioxidants Vitamins A or C in this study were affected by the flaxseed supplementation over the study period of 12 weeks.

Studies by Sundaram *et al.,* (1996), Mattia *et al.,* (2003) and Opara *et al.,* (1999) have reported an increase in peroxidation products in subjects with T2DM with varying degree of complications and duration of disease. Prasad (2001) also suggested that T2DM is associated with oxidative stress, as he reported lower MDA levels in SDG treated ZDF rats that did not develop diabetes compared to the groups of ZDF rats that were untreated with SDG and were allowed to develop diabetes. Our study showed no change in the plasma hydroperoxide levels in the flaxseed treated groups and the control group, which are in line with the results of Cunnane *et al.,* (1994) where 50 gm flaxseed/day was given to healthy young adults. When looking at the time effect, plasma hydroperoxide levels were slightly low at 4, 8 and 12 weeks when compared to the control group indicating that long term consumption of flaxseed may have effect on oxidative stress, however all the levels are within the normal range of plasma hydroperoxide (0.22 – 7.8 μM).

A main significant group effect was seen, revealing that the urinary isoprostane levels in the control group were significantly higher then the milled flax and flax oil group over the period of study. There was no significant effect seen on the groups consuming either milled flax or flax oil. The isoprostane levels in the control group were higher at all time point. None of the published studies has observed the effect of flaxseed supplementation on F_2-isoprostanes, among the individuals with metabolic syndrome.

Examination of changes in plasma protein carbonyl in week 0 and 12 revealed no significant differences by either treatment or time (Patel *et al.,* 2004). Odetti *et at.,* (1999) reported that the determination of protein carbonyl groups, introduced by Stadtman ER, has been proposed as a stable marker of oxidative damage of proteins. Ruhe & McDonald (2001) and Bonnefont-Rousselot (2002), have reported that oxidative stress resulting from hyperglycemia has been suggested to play a role in T2DM, and that hyperglycemia is commonly cited as the basis for oxidative stress in diabetes. According to Cockell (unpublished data), i*n vitro* incubation of albumin with high glucose increased the carbonyl content of the albumin.

Oxidative stress parameters such as plasma protein carbonyls might be expected to show much less change if normoglycemia is maintained through medical intervention. According to Halliwell & Gutteridge (1999), hyperglycemia results in non-enzymatic glycosylation of proteins, including conversion of haemoglobin to HbA1C. Amadori products intermediate in this process includes reactive carbonyl groups, though these may no longer be present in subsequent advanced glycation end products. Cockell (unpublished data) concluded that, if diabetes is kept under control like the subjects in this study, with glycemia maintained, it is not clear whether significant accumulation of protein carbonyls would be detected which might be the case in this study. Odetti *et al.,* (1999) stated that the level of plasma carbonyl content in diabetic subjects were slightly, but not significantly, higher than controls, suggesting that the damage on circulating proteins is strictly controlled by means of their catabolism. Odetti *et al.*, (1999) also reported a significant inverse correlation between carbonyl groups and plasma proteins; they supposed that the extensively damaged proteins are quickly removed from the blood,

supporting a rigid control of the injury degree. Overall, in this study no significant difference was seen in the protein carbonyl content among the groups, either by time or by treatments at 0 and 12 weeks.

Erythrocyte enzymatic antioxidant activitiy has been reported to be affected in T2DM. Comparing with other studies reported, Sundaram *et al.,* (1996) and Parthiban *et al.,* (1995) reported that erythrocyte GPx activity was significantly elevated whereas CAT and SOD activity decreased in diabetic subjects within 2 years of the onset of the disease. SOD activity was slightly but not significantly decreased in subjects with T2DM whereas Akkus *et al.,* (1996) reported no change in leukocyte GPx activity. Kesavulu *et al.,* (2001) reported an increase in erythrocyte CAT activity, decrease in GPx activity and no change in the SOD activity in both groups of diabetic patients and non-diabetic patients with coronary heart disease (CHD) compared to controls. Memisogullari *et al.,* (2003) observed similar changes with erythrocyte enzymes where CAT activity was increased, no change was observed in SOD activity and GPx activity was decreased in T2DM subjects compared to controls.

None of the studies published with flaxseed supplementation in humans has investigated erythrocyte SOD, CAT and GPx enzymatic activity. Our results did not show any change in erythrocyte SOD and CAT activity over the time in any of the groups. A significant time×group (time and group interaction) was seen on both the enzymes indicating that there may be some effect of the flaxseed on subjects over the period of 12 weeks. Both erythrocyte CAT and SOD activity were higher in the milled flax group at week 0. Erythrocyte CAT activity was higher at week 4 in the flax oil group and the Vitamin C level at week 4 in the flax oil group was lower than the control group

which may be indicative of the presence of increase in free radicals. Total antioxidant property determined by FRAP showed no change in the milled flax and the flax oil groups compared to the control group. A significant main time effect was observed showing that the total antioxidant property were lower at other time points when compared to 0 week, which may be a sign of the increase in activity of antioxidants against free radicals. However, all the levels were within the normal range. Our views agree with the previous report of Cunnane *et al.,* (1994) on the lack of change in these same variables during flaxseed supplementation and that relatively large amounts of flaxseed given as dietary supplement do not cause an obvious change in antioxidant status.

These results corroborate with the previous findings by Prasad (2001) that SDG may be most important in interrupting a pathologic process, rather than its effect when there is no pathologic condition. The long-term use of SDG would have antidiabetic influence in those with slight tendency in that direction and especially in those who have family history of diabetes (Prasad, 2001).

One of the possible causes of no change in any of the biochemical parameters measured with dietary treatment could be that these subjects did not have advanced pathologic condition or any severe complications that increased the production of free radicals. Hence, despite the higher intake of α-linolenate in any form i.e. milled flaxseed or flax oil, it did not appear to affect the indices of lipid peroxidation (hydroperoxides and 8- isoprostanes), protein carbonyls, Vitamin antioxidants A, E, and C, as well as no effect was seen on the enzymatic antioxidants SOD and CAT. Slight variations observed in few of the parameters measured at 0, 4, 8 and 12 weeks could be due to a

compensation mechanism of the body, environmental factors or dietary pattern of an individual. Unfortunately, for this part of the study, dietary assessments of these subjects were not observed.

Our subjects were controlled diabetics. More over these subjects tended to have metabolic syndrome as defined by the World Health Organization (WHO) and National Cholesterol Education Program (NCEP) rather than having T2DM. These subjects were diagnosed with T2DM when recruited for the study as intended. But after screening and assessing the biochemical parameters like blood glucose, glycated haemoglobin, triglycerides, HDL-C, LDL-C and BMI, we found they were falling into the criteria of having metabolic syndrome and that these parameters do not support that these subjects had T2DM.

The metabolic syndrome is a multifaceted clinical entity resulting from the interaction of genetic, hormonal, and lifestyle factors. The metabolic syndrome has become a powerful predictor of disease and its diagnosis can be used as a valuable preventive tool. A clustering of risk factors, including hyperinsulinaemia, dyslipidaemia, hypertension and glucose intolerance (rather than hyperinsulinaemia alone), are characterized as the underlying features of the metabolic syndrome and also advocate the name "insulin resistance syndrome" as it is caused by insulin resistance (Boehm & Boehm, 2005).

According to the National Cholesterol Education Program (NCEP): Adult Treatment Panel (ATP) III, the metabolic syndrome is defined as 3 or more of the following: (Boehm & Boehm, 2005).

Central obesity: Waist circumference >102 cm (male), >88 cm (female)

Hypertriglyceridaemia: triglycerides ≥ 1.7 Mmol/L

Low HDL-C: <1.0 Mmol/L (male), <1.3 Mmol/L (female)

Hypertension: blood pressure ≥ 135/85 mm Hg and/or medication

Fasting plasma glucose: ≥ 6.1 Mmol/L

And according to World Health Organization (WHO), metabolic syndrome is defined as diabetes or impaired fasting glycaemia or impaired glucose tolerance or insulin resistance plus 2 or more of the following (Bernhard *et al.,* 2005):

Obesity: BMI > 30 or waist-to-hip ratio > 0.9 (male) > 0.85 (female)

Dyslipidaemia: triglycerides ≥ 1.7 Mmol/L or HDL-C <0.9 (male) or <1.0 (female) Mmol/L

Hypertension: blood pressure > 140/90 mmHg and/or medication

Microalbuminuria: albumin excretion > 20 μg/min

According to these criteria, our subjects had metabolic syndrome.

One of the objectives of the study was to measure GPx antioxidative enzyme present in our body. As none of the treatment groups showed any effect of flaxseed supplementation on any of the biochemical parameters measured, especially SOD and CAT enzymes, we decided not to measure the activity of GPx in erythrocytes.

CHAPTER NINE - CONCLUSIONS

Flaxseed supplementation either as milled flaxseed or flax oil (7.5 gm ALA) in food products did not had any effect on the oxidative status of these subjects. No alterations were observed in the biochemical parameters measured. Flaxseed did not increase or decrease the indices of lipid peroxidation in these subjects. Antioxidant enzymes such as erythrocyte SOD and CAT activity were unaltered as well as the total antioxidant property was not affected in these subjects after consuming flaxseed for 12 weeks. In addition, these subjects had metabolic syndrome and not T2DM.

Again, focus should be directed on the possibility that flaxseed may be important or effective in interrupting the pathologic process rather then when there is no pathologic condition present. While this was our intent, the subjects did not fit the former category.

CHAPTER TEN – STRENGTHS AND LIMITATIONS

STRENGTHS:

- Addresses questions with regards to the consumption of milled flax and flax oil on antioxidant status in individuals with metabolic syndrome.
- To my knowledge this is the first study measuring all the following parameters after consumption of flax products to see its effect on oxidant status:
  - i) enzymatic antioxidants (erythrocyte SOD & CAT)
  - ii) non-enzymatic antioxidants (Vitamins A, E and C)
  - iii) total antioxidant property, and
  - iv) lipid peroxidation indices: isoprostanes, protein carbonyls and hydroperoxides
- Samples were collected at four different time points over the 12 weeks for each individual which gives repetitive values for each biochemical parameters measured.
- Subjects in this study actually consumed flax products instead of supplements in other form like capsules etc.
- Human study.

LIMITATIONS:

- Subjects were not having acute or chronic T2DM.
- Normal healthy control group was not included in the study.
- Antioxidant enzyme GPx was not measured.

CHAPTER ELEVEN – AREAS FOR FUTURE RESEARCH

1. Effect of flaxseed supplementation in any other form (ground or defatted) on the antioxidative status; as studies have shown varying results with the use of flaxseed.
2. Supplementation of milled flaxseed to subjects with acute or chronic diabetes or other pathologic conditions to see the effects of lignans on oxidative status.
3. Antioxidant Vitamins and enzymes should be studied closely may be at cellular level after flaxseed supplementation.
4. See effects of supplementing isolated form of SDG on the indices of lipid peroxidation.

REFERENCES

Aebi H. Catalase *in vitro. Methods in Enzymology,* 105, 1984, 121-126.

Al-Talla Z A and Tolley L T. Analysis of Vitamin E derivatives in serum using coordinated ion spray mass spectrometry. *Rapid Communications in Mass Spectrometry,* 19, 2005, 2337-2342.

Babu S. U, Mitchell G V, Wiesenfeld P, Jenkins M Y and Gowda H. Nutritional and hematological impact of dietary flaxseed and defatted flaxseed meal in rats. *International Journal of Food Science and Nutrition,* 51, 2000, 109-117.

Akkus I, Kalak S, Vural H, Caglayan O, Menekse E, Can G and Durmus B. Leukocyte lipid peroxidation, superoxide dismutase, glutathione peroxidase and serum & leukocyte Vitamin C level of patients with type 2 diabetes mellitus. *Clinica Chimica Acta,* 244, 1996, 221-227.

Benzie I and Strain J. Ferric reducing antioxidant power assay. Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of a total antioxidant power and ascorbic acid concentration. *Methods in Enzymology,* 299, 1999, 15-27.

Boehm B. O and Boehm S. C. The Metabolic Syndrome. *Scandinavian Journal of Clinical and Laboratory Investigation,* 65 (S240), 2005, 3-13.

Bonnefont-Rousselot D. Glucose and reactive oxygen species. *Current Opinion in Clinical Nutrition and Metabolic Care,* 5, 2002, 561-568.

Carmeli E, Coleman R and Berner Y. Activities of antioxidant scavenger enzymes (superoxide dismutase and glutathione peroxidase in erythrocytes in adult women with and without type II diabetes. *Experimental Diabetic Research,* 5, 2004, 171-175.

Connor W E, Prince M J, Ullmann D, Riddle M, Hatcher L, Smith F E and Wilson D. The hypotriglyceridemic effect of fish oil in adult onset diabetes without adverse glucose control. *Annals of New York Academy Sciences,* 683, 1993, 337-340.

Cunnane S C, Hamadeh M J, Liede A C, Thompson L U, Wolever T M S and Jenkins D J A. Nutritional attributes of traditional flaxseed in healthy young adults. *American Journal of Clinical Nutrition,* 61, 1995, 62-68.

Cunnane S C, Ganguli S, Menard C, Liede A C, Hamadeh M J, Chen Z Y, Wolever T M S and Jenkins D J A. High α-linolenic acid flaxseed (*Linum usitatissimum*): some nutritional properties in humans. *British Journal of Nutrition,* 69, 1993, 443-453.

Devaraj S, Hirany S V, Burk R F and Jialal I. Divergence between LDL oxidative susceptibility and urinary F_2-isoprostanes as measures of oxidative stress in type 2 diabetes. *Clinical Chemistry,* 47(11), 2001, 1974-1979.

Driskell W J, Neese J W, Bryant C C and Bashor M M. Measurement of Vitamin A and Vitamin E in human serum by high-performance liquid chromatography. *Journal of Chromatography*, 231, 1982, 439-444.

Farkas K, Sasvari M, Szaleczki E, Pusztai P and Somogyi A. Erythrocyte antioxidant status of type 2 diabetic patients with and without complications. 2nd Department of Medicine, Semmelweis University of Medicine; Central Research Laboratory, Haynal Imre University of Health Sciences, Budapest, Hungary.

Farrell P M, Levine S L, Murphy M. D and Adams A J. Plasma tocopherols levels and tocopherol-lipid relationships in a normal population of children as compared to healthy adults. *American Journal of Clinical Nutrition,* 31, 1978, 1720-1726.

Flax Council of Canada. Flaxseed and Human Nutrition. 465-167 Lambard Ave. Winnipeg, MB, Canada R3B 0T6. www.flaxcouncil.ca.

Fletcher B, Gulanick M and Lamendola C. Risk factors for type 2 diabetes. *Journal of Cardiovascular Nursing,* 16(2), 2002, 17-23.

Frankel E N. Lipid Oxidation: University of California, Davis, California, USA. The Oily Press Dundee, (10), 1998. 6 Dunnottar Place, West Ferry, Dundee. Scotland DD5 1PJ. Printed in Great Britain by Bell and Bain Ltd. Glasgow.

Frenich A G, Torres M E H, Vega A B, Vidal J L M and Bolanos P P. Determination of ascorbic acid and carotenoids in food commodities by liquid chromatography with mass spectrometry detection. *Journal of Agricultural and Food Chemistry*, 53, 2005, 7371-7376.

Friel J, Friesen R, Harding S and Roberts L. Evidence of oxidative stress in full-term healthy infants. *Pediatric Research,* 56(6), 2004, 878-882.

Gallan P, Carrascosa A, Gussinye M and Dominguez C. Biomarkers of diabetes – associated oxidative stress and antioxidant status in young diabetic patients with or without subclinical complications. *Free Radical Biology and Medicine,* 34(12), 2003, 1563-1574.

Giugliano D, Ceriello A and Paolisso G. Oxidative stress and diabetic vascular complications. *Diabetes Care,* 19(3), 1996, 257-267.

Grisham M B. Reactive Metabolites of oxygen and nitrogen in biology and medicine. Medical Intelligence Unit Publications. CRC Press, 2000 Corporate Blvd., NW, Boca Raton, FL 33431,1994.

Haag M._Essential fatty acids and the brain. *The Canadian Journal of Psychiatry*, 48, 2003, 195-203.

Halliwell B. Vitamin E and the treatment and prevention of diabetes: A case for a controlled clinical trial. *Singapore Medical Journal,* 43(9), 2002, 479-484.

Halliwell B and Gutteridge J M C. Free Radicals in Biology and Medicine. Oxford University Press, Inc., New York.

Herbeth B, Zittoun J, Miravet L, Bourgeay-Causse M, Carre-Guery G, Delacoux E, Devehat C L, Lemoine A, Mareschi J P, Martin J, Potier de Courcy G and Sancho J. Reference intervals for Vitamins B1, B2, E, D, retinol, beta-carotene and folate in blood: Usefulness of dietary selection criteria. *Clinical Chemistry,* 32 (9), 1986, 1756-1759.

Indu M and Ghafoorunissa. N-3 fatty acids in Indian diets comparison of the effects of precursor (α-linolenic acid) vs product (long chain n-3 polyunsaturated fatty acids). *Nutrition Research,* 12, 1992, 569-82.

Jakus V. The role of free radicals, oxidative stress and antioxidant systems in diabetic vascular disease. *Brastisl Lek Listy,* 101(10), 2000, 541-551.

Jenkins D, Kendall C, Vidgen E, Agarwal S, Rao A V, Rosenberg R S, Diamandis E P, Novokmet R, Mehling C C, Perera T, Griffin L C and Cunnane S C. Health aspects of partially defatted flaxseed, including effects on serum lipids, oxidative measures, and ex vivo androgen and progestin activity: a controlled crossover trial. *American Journal of Clinical Nutrition,* 69, 1999, 395-402.

Kanauchi M, Kawano T, Kanauchi K and Saito Y. New "prediabetes" category and the metabolic syndrome in Japanese. *Hormonal Metabolic Research,* 37, 2005, 622-626.

Kesavulu M M, Giri R, Kameswara Rao B and Apparao C. Lipid peroxidation and antioxidant enzymes levels in type 2 diabetics with microvascular complications. *Diabetes Metabolism,* 26(5), 2000, 387-92.

King G L and Loeken M R. Hyperglycemia-induced oxidative stress in diabetic complications. *Histochemistry and Cell Biology,* 122, 2004, 333-338.

Kitts D D, Yuan Y V, Wijewickreme A N and Thompson L U. Antioxidant activity of the flaxseed lignan secoisolariciresinol diglycoside and its mammalian lignan metabolites enterodiol and enterolactone. *Molecular and Cellular Biochemistry,* 202, 1999, 91-100.

Lee P and Prasad K. Effects of flaxseed oil on serum lipids and atherosclerosis in hypercholesterolemic rabbits. *Journal of Cardiovascular Pharmacology and Therapeutics*, 8(3), 2003, 227-235.

Marklund S, Westman N, Lundgren E and Roos G. Enzyme defenses in normal and neoplastic cells and tissues. *Cancer Research,* 42, May 1982, 1955-1961.

Mattia G D, Laurenti O and Fava D. Diabetic endothelial dysfunction, effect of free radical scavenging in type 2 diabetic people. *Journal of Diabetes and its Complications*, 17, 2003, 30-35.

Memisogullari R, Taysi S, Bakan E and Capoglu I. Antioxidant status and lipid peroxidation in type II diabetes mellitus. *Cell Biochemistry and Function,* 21, 2003, 291-296.

Mezzetti A, Cipollone F and Cuccurullo F. Oxidative stress and cardiovascular complications in diabetes: isoprostances as new markers on an old paradigm. *Cardiovascular Research,* 47, 2000, 475-488.

Miyata T, Inagi R, Asahi K, Yamada Y, Horie K, Sakai H, Uchida K and Kurokawa K. Generation of protein carbonyls by glycoxidation and lipoxidation reactions with autoxidation products of ascorbic acid and polyunsaturated fatty acids. *FEBS Letters* 437, 1998, 24-28.

Morrow J D and Roberts L J. The isoprostanes. Their role as an index of oxidant stress status in human pulmonary disease. *American Journal of Respiratory and Critical Care Medicine,* 166, 2002, 25S-30S.

Nourooz-Zadeh J, Rahimi A, Sarmadi J T, Tritschler H, Rosen P, Halliwell B and Betteridge D J. Relationships between plasma measures of oxidative stress and metabolic control in NIDDM. *Diabetologia,* 40, 1997, 647-653.

Nourooz-Zadeh J, Tajaddini Sarmadi J and Wolff S F. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation xylenol orange assay in conjunction with triphenylphosphine. *Analytical Biochemistry,* 220, 1994, 403-409.

Nourooz-Zadeh J, Tajaddini Sarmadi J, McCarthy S, Betteridge D J and Wolff S P. Elevated levels of authentic plasma hydroperoxides in NIDDM. *Diabetes,* 44, 1995, 1054-1058.

Odetti P, Garibaldi S, Noberasco G, Aragno I, Valentini S, Traverso N and Marinari U. Levels of carbonyl groups in plasma proteins of type 2 diabetes mellitus subjects. *Acta Diabetologica,* 36, 1999, 179-183.

Omaye S T, Turnbull J D and Sauberlich H E. Selected methods for the determination of ascorbic acid in animal cells, tissues, and fluids. *Methods in Enzymology,* 62, 1979, 3-11.

Oomah B D. Flaxseed as a functional food source. *Journal of the Science of Food and Agriculture,* 81, 2001, 889-894.

Opara E C, Abdel-Rahman E, Soliman S, Kamel W A, Souka S, Lowe J E and Abdel-Aleem S. Depletion of total antioxidant capacity in type 2 diabetes. *Metabolism,* 48(11), 1999, 1414-1417.

Packer L, Traber M G, Kraemer K and Frei B. The antioxidant Vitamins C and E. AOCS Press, Champaign, Illinois, 2002.

Panda K, Chattopadhyay R, Chaattopadhyay D J and Chatterjee I B. Vitamin C prevents cigrattee smoke induced oxidative damage *in vivo*. *Free Radical Biology and Medicine,* 29(2), 2000, 115-124.

Paolisso G and Giugliano D. Oxidative stress and type 2 diabetes mellitus. *International Diabetes Monitor,* 10, (2), 1998, 1-6.

Patel T, Taylor C G, Cockell K A and Friel J K. Oxidative status of individuals with type 2 diabetes before, during and after comsumption of milled flaxseed and flaxseed oil as compared to controls. First Northern Lights Summer Conference coordinated by Canadian Federation of Biological Societies. 47th Annual Meeting, 2004.

Parthiban A, Vijayalingam S, Shanmugasundaram R K and Mohan R. Oxidative stress and the development of diabetic complication-antioxidants and lipid peroxidation in erythrocytes and cell membrane. *Cell Biology International,* 19(12), 1995, 987-993.

Patrono C and Fitzgerald G A. Isoprostanes: Potential markers of oxidant stress in atherothrombotic disease. *Arteriosclerosis, Thrombosis and Vascular Biology,* 17, 1997, 2309-2315.

Prasad K. Secoisolariciresinol diglucoside from flaxseed delays the development of type 2 diabetes in Zucker rat. *Journal of Laboratory and Clinical Medicine,* 138(1), 2001, 32-9.

Prasad K. Oxidative stress as a mechanism of diabetes in diabetic BB prone rats: Effect of secoisolariciresinol diaglucoside (SDG). *Molecular and Cellular Biochemistry,* 209, 2000, 89-96.

Puhakainen I, Ahola I and Yki-Jarvinen H. Dietary supplementation with n-3 fatty acids increases gluconeogenesis from glycerol but not hepatic glucose production in patients with non-insulin-dependent diabetes mellitus. *American Journal of Clinical Nutrition,* 61, 1995, 121-126.

Quinn L. Mechanisms in the development of type 2 diabetes mellitus. *Journal of Cardiovascular Nursing,* 16(2), 2002, 1-16.

Roberts L J, II and Morrow J D. Measurement of F_2-isoprostanes as an index of oxidative stress *in vivo. Free Radical Biology & Medicine*, 28(4), 2000, 505-513.

Ruhe R C and Mc Donald R B. Use of antioxidants nutrients in the prevention and treatment of type 2 diabetes. *Journal of American College of Nutrition*, 20, 2001, 363S-369S.

Ruiz C, Alegria A, Barbera R, Farre R and Lagarda M J. Lipid peroxidation and antioxidant enzymes activities in patients with type 1 diabetes mellitus. *Scandinavian Journal of Clinical Laboratory Investigation,* 59(2), 1999, 99-105.

Sekeroglu M R, Sahin H, Dulger H and Algun E. The effect of dietary treatment on erythrocyte lipid peroxidation, superoxide dismutase, glutathione peroxidase and serum lipid peroxidation in patients with type 2 diabetes mellitus. *Clinical Biochemistry,* 33(8), 2000, 669-674.

Simopoulos A P. Essential fatty acids in health and chronic disease. *American Journal of Clinical Nutrition,* 70(suppl): 1999, 560S-9S.

Sorrentino M J. Implications of the Metabolic Syndrome: the New Epidemic. *American Journal Cardiology,* 96(suppl), 2005, 3E-7E.

Sozmen E Y, Sozmen B, Delen Y and Onat T. Catalase/superoxide Dismutase and catalse/paraoxonase ratios may implicate poor glycemic control. *Archives of Medical Research,* 32, 2001, 283-287.

Spitz R D and Oberley W L. Current Protocols in Toxicology-Unit 7.6. Measurement of MnSOD and CuZnSOD activity in mammalian tissue homogenates. Free Radical and Radiation Biology Program, B180 medical Laboratories, Department of Radiology University of Iowa, Iowa City, Iowa 52242.

Stahl W and Sies H. Antioxidant defense. Vitamins E & C and Carotenoids. *Diabetes,* 46, suppl.2, 1997, 14S-18S.

Sundaram R K, Bhaskar A, Vijayalingam S, Viswanathan M, Mohan R and Shanmugasundaram K R. Antioxidant status and lipid peroxidation in type II diabetes mellitus with and without complications. *Clinical Science,* 90, 1996, 255-260.

Turk H M. Plasma lipid peroxidation products and antioxidant enzyme activities in patients with type 2 diabetes mellitus. *Acta Diabetologica,* 39, 2002, 117-122.

West I C. Radicals and oxidative stress in diabetes. *Diabetic Medicine,* 17, 2000, 171-180.

Wiesenfeld P W, Babu U S, Collins T F X, Sprando R, O'Donnell M W, Flynn T J, Black T and Olejnik N. Flaxseed increased alpha linolenic and eicosapentaenoic acid and decreased arachidonic acid in serum and tissues of rat dams and offspring. *Food and Chemical Toxicology,* 41, 2003, 841-855.

Wilson P W F, Agostino R B D, Parise H, Sullivan L, and Meigs J B. Metabolic syndrome as precursor of cardiovascular disease and type 2 diabetes mellitus. *Circulation,* 112, 2005, 3066-3072.

APPENDICES

1. Measuring total antioxidant property in plasma:

Method: By Benzie I F F and Strain J J., 1999.

Principle:

At low pH, reduction of a ferric tripyridyltriazine (Fe^{III}-TPTZ) complex to the ferrous form, which has an intense blue color, can be monitored by measuring the change in absorption at 593 nm. The reaction is nonspecific, in that any half reaction that has lower redox potential, under reaction conditions, than the ferric/ferrous half reaction will drive the ferric (Fe^{III}) to ferrous (Fe^{II}) reaction.

Chemicals:

1. Sodium Acetate Trihydrate
2. Glacial Acetic Acid
3. 2,4,6-Tripyridyl-s-triazine (TPTZ)
4. Hydrochloric Acid (HCl)
5. Ferric Chloride Hexahydrate ($FeCl_3 .6H_2O$)
6. Ferrous sulphate ($FeSO_4$)

Reagents:

1. Acetate buffer (300 Mmol) (pH 3.6): 3.1 gm sodium acetate trihydrate and 16 ml glacial acetic acid made up to 1 liter with distilled water.
2. Working FRAP Reagent: Mix 10 Mmol (TPTZ) 2,4,6-Tripyridyl-s-triazine, 40 Mmol HCl and 20 Mmol Ferric Chloride Hexahydrate ($FeCl_3 .6H_2O$) in ratio of 10:1:1.

Procedure:

1. Prepare 50,000 µM/3.1 ml stock of ferrous sulphate.
2. Prepare the following concentrations:

Conc. (µM)	From	Take (ul)	Add D.W (ul)	Total vol. (ul)
200	50,000 µM	12.4	3087.6	3100
500	50,000 µM	31	3069	3100
1000	50,000 µM	62	3038	3100
1500	50,000 µM	93	3007	3100
2000	50,000 µM	124	2976	3100

3. **For Standards**: Take 100 ul of above standards and add 3 ml of FRAP reagent. Vortex. Read absorbance at 593 nm against a reagent blank at a predetermined time after sample reagent mixing.
4. Since test was performed at room temperature, a 0 to 6 minutes reaction time window is preferable as the reaction of the uric acid is slightly slower at lower temperature.
5. **For Samples**: Take 100 ul of plasma and add 3 ml of FRAP reagent. Vortex. Read absorbance at 593 nm against a reagent blank at a predetermined time after sample reagent mixing.
6. **For Blank**: 100 ul of distilled water and 3 ml of FRAP reagent.
7. Milton Roy (spectronic 3000 array) spectrophotometer was used for reading the absorbance.

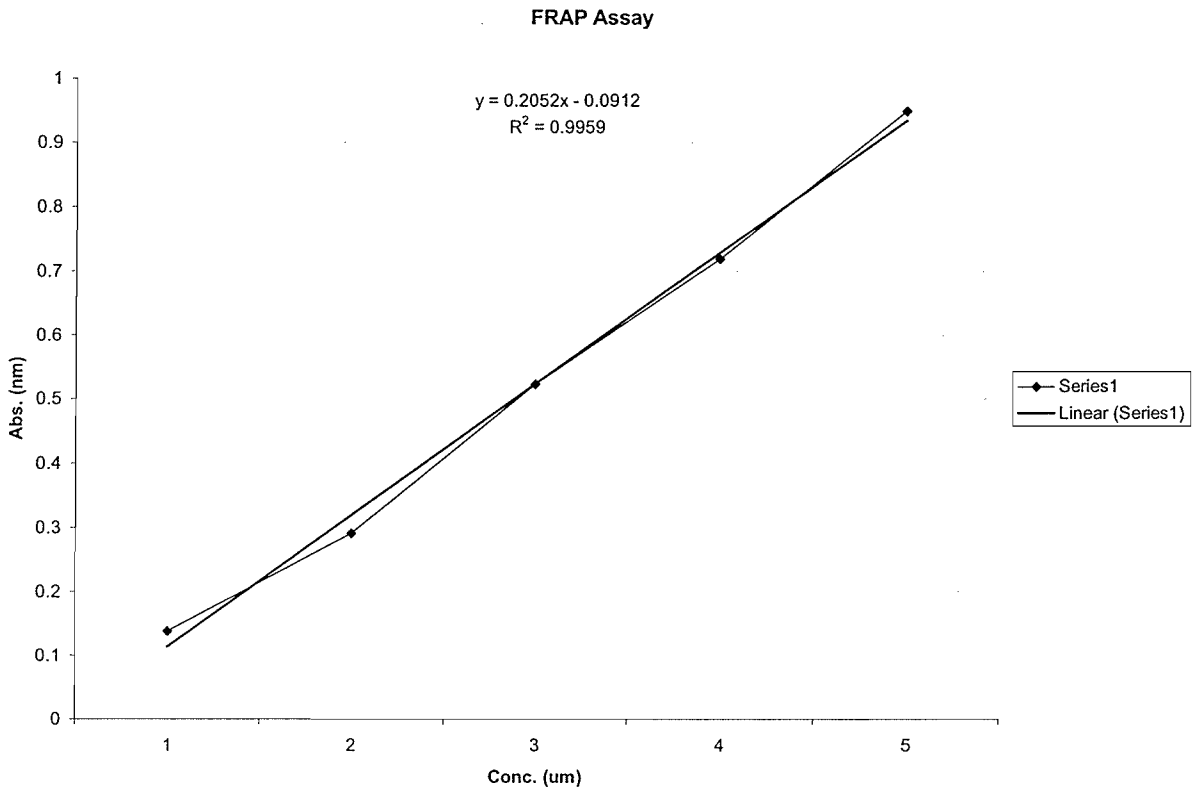


Figure 31. Standard curve for FRAP assay.

Result:

Absorbance change is translated into a FRAP value (μM) by relating the absorbance value of test sample to that of standard solution of known FRAP value:

$$\frac{\text{0-6 min absorbance test sample}}{\text{0-6 min absorbance standard}} \times \text{FRAP value of standard (μM)}$$

2. Measuring Vitamin C in plasma:

Method: By Omaye S T *et al.,* 1979.

Reagents:

1. 5% Trichloroacetic Acid (TCA): Weigh 12.5 gm and dissolve in 250 ml distill water.

2. DTC Solution 2,4-dinitrophenylhydrazine (DNPH)/Thiourea/Copper: Weigh 0.75 gm DNPH, 0.1 gm thiourea and 0.0125 gm copper sulphate. Dissolve in 25 ml of 9N sulphuric acid.

3. 9N Sulphuric acid: 25 ml sulphuric acid and add 50 ml "ice cold" distilled water in a 100 ml flask and cool overnight, and then bring to 100 ml with "ice cold" distilled water.

4. 65% Sulphuric acid: 65 ml sulphuric acid and add 25 ml "ice cold" distilled water in a 100 ml flask and allow to cool overnight, and then bring to 100 ml with distilled water.

5. Standards:

(a) Stock (1 mg/ml): Weigh 50 mg ascorbic acid and bring to 50 ml with 5% TCA.

(b) Working Stock (0.1 µg/ul): Take 400 ul stock and add 3.6 ml of 5% TCA.

Procedure:

1. Prepare the standards as follows:

Conc. (µg/ml)	From working Stock (ul)	Add 5% TCA (ul)	Final vol. (ul)
blank	-	1000	1000
0.5	5	995	1000
1.5	15	985	1000
25	25	975	1000
3.5	35	965	1000
4.5	45	955	1000
20	200	800	1000

2. **For Samples:** Take 100 ul plasma and add 900 ul of "ice cold" 5% TCA. Vortex and centrifuge for 20 minutes at 3500g. Take 300 ul x 3 of supernatant. Add 100 ul DTC, and vortex. Incubate at 37°C in hot water bath for 3 hours. Add 500 ul of "ice cold" 65% sulphuric acid then incubate at room temperature for 30 minutes and read at 520 nm.

3. **For Standards:** Take 300 ul x 3 of standards and follow the sample procedure.

4. Milton Roy (spectronic 3000 array) spectrophotometer was used for reading the absorbance.

Calculations:

To find the interpolated value plot the absorbance values of the standards versus their corresponding Vitamin C concentrations.

Vitamin C (mg/dl) = Interpolated value x 10

3. Measuring Vitamin A and E in plasma:

Method: By Driskell *et al.,* 1982.

Chemicals and Equipment:

1. Running solvent-Methanol 100% HPLC grade
2. Ethanol 100% HPLC grade
3. α-tocopherol
4. Retinol Acetate
5. All trans-Retinol
6. Hexane
7. Chloroform

8. HPLC Beckman System Gold (solvent module 126, detector module 166 and integrator 3390A)
9. Injection syringe 50 ul
10. Column C18

Reagents:

1. Retinol acetate:
   (a) 4000 µg/ml- Weigh 0.202 gm and make up to 50 ml with ethanol.
   (b) 1000 µg/ml- Take 6.25 ml from 4000 µg/ml and make up to 25 ml with ethanol.
   (c) 20 µg/ml- Take 1 ml from 1000 µg/ml and make up to 50 ml with ethanol.
2. All trans retinol (Vitamin A):
   (a) 500 µg/ml- Weigh 0.0125 gm, all trans retinol and make up to 25 ml with ethanol.
   (b) 50 µg/ml- Take 2.5 ml from 500 µg/ml and make up to 25 ml with ethanol.
3. α-Tocopherol (Vitamin E):
   (a) 500 µg/ml- Weigh 0.025 gm α-tocopherol and make up to 50 ml with ethanol.
   (b) 200 µg/ml- Take 20 ml from 500 µg/ml and make up to 50 ml with ethanol.

Procedure:

1. **Standards:** Take five 25 ml volumetric flasks and add retinol acetate, α-tocopherol and all trans retinol in following amount and bring the volume to 25 ml with methanol.

For Vitamin A:

Volume taken (ml) from 50 µg/ml	Flask vol. (ml) (methanol)	Vitamin A in flask (µg)	Conc. desired (µg/ml)	µg Vitamin A with 50 ul inj.
0.1	25	5	0.2	0.01
0.2	25	10	0.4	0.02
0.3	25	15	0.6	0.03
0.4	25	20	0.8	0.04
0.5	25	25	1	0.05

For Vitamin E:

Volume taken (ml) from 200 µg/ml	Flask vol. (ml) (methanol)	Vitamin E in Flask (µg)	Conc. desired (µg/ml)	µg Vitamin E with 50 ul inj.
0.5	25	100	4	0.2
1	25	200	8	0.4
1.5	25	300	12	0.6
2.25	25	450	18	0.9
3	25	600	24	1.2

For Retinal Acetate:

Volume taken (ml) from 20 µg/ml	Flask vol. ml (methanol)	Retinal Acetate in flask (µg)	Conc. desired (µg/ml)	ug Retinal Acetate with 50 ul inj
1.25	25	25	1	0.05
1.25	25	25	1	0.05
1.25	25	25	1	0.05
1.25	25	25	1	0.05
1.25	25	25	1	0.05

2. **Samples:** Take 100 ul of plasma in centrifuge tube and add 10 ul of retinal acetate and 90 ul of ethanol, vortex for 20 seconds. Add 200 ul of hexane and vortex for 30 seconds. Centrifuge for 1 minute at 1000g. Take 150 ul of hexane from the supernatant in another centrifuge tube. Evaporate hexane layer under nitrogen (clean nitrogen needles with chloroform before drying). Add 150 ul of ethanol from sides of the centrifuge tube. Filter the samples and inject 50 ul twice.

Calculations:

To find the interpolated value plot the absorbance values of the standards versus their corresponding Vitamin E and A concentrations respectively.

Vitamin E and A (μg/ml) = Interpolated value x 20

4. Measuring catalase activity in erythrocytes:

Method: By Aebi H, 1984.

Reagents: Make fresh every day if possible. Store at 2°C.

1. Assay Buffer (phosphate buffer): 5 Mmol pH 7.0

a) 6.81 gm potassium dihydrogen (KH_2PO_4) in 1 litre water

b) 8.9 gm sodium hydrogen phosphate ($Na_2HPO_4.2H_2O$) in 1 litre water.

Mix solution a and b in 1:1.5 ratio v/v.

2. Hydrogen peroxide (H_2O_2): 30 Mmol.

Dilute 0.34 ml of 30% H_2O_2 with phosphate buffer to 100 ml.

Procedure:

Sample Preparation (Erythrocyte):

a) When frozen samples are used, 10 ul of packed red cells are diluted to 20 ml with phosphate buffer. Mix gently.

b) Reference cuvette: 500 ul phosphate buffer + 1 ml sample

c) Test cuvette: 1 ml sample + 500 ul H_2O_2

The spectrophotometer (ultrospec 4000-Pharmacia Biotech) is set at 240 nm for measuing the reaction kinetics. To test cuvette 500 ul of H_2O_2 is added to a cuvette already placed in the spectrophotometer and containing the sample. The cuvette is quickly mixed by pipette while pipetting and change in absorbence is recorded for four miinutes.

Calculations:

Catalase specific activity at 25°C is defined as Mmole of H_2O_2 consumed/min./mg protein in sample. The conversion of initial velocity (difference in absorbance) to catalase specific activity is as follows:

Specific activity (units/mg protein):

1. Difference in absorbance (A_I-A_F)/min at 240 nm × Total dilution factor × 34403/1000 = Result 1 (U/ml)

Where, 34403 is derived by following formula:

Factor = total vol × 10^6/molar extinction coefficient of hydroperoxide × path length × sample volume

2. mg protein/ml (from protein estimation) = Result 2

3. Result 1 / Result 2 = Catalase specific activity at 25°C (units/mg protein)

5. Estimation of protein in RBC:

Method: Sigma Chemicals (Product codes TP0300 and L540) total protein kit.

Reagents:

1. Lowry Reagent Solution: Lowry's reagent is prepared by adding 40 ml of water to a bottle of Lowry reagent powder. Mix well by inverting to completely dissolve the contents. Do not shake to minimize foaming.

2. The Folin and Ciocalteu's Phenol Reagent: The working solution is prepared by transferring the Folin and Ciocalteu's phenol reagent (18 ml) to the amber glass bottle provided. Rinse the Folin and Ciocalteu's phenol reagent bottle with 10 ml of water and add the rinse solution to the working solution bottle. Add an additional 80 ml of water to the working solution bottle and mix well. Store the Folin and Ciocalteu's phenol reagent working solution at room temperature.

3. Protein Standard: Protein standard solution (400 µg/ml) is prepared by adding appropriate volume of water to the vial. Swirl gently to completely dissolve the contents.

4. Store the Lowry reagent solution and the Folin and Ciocalteu's phenol reagent at room temperature. Do not refrigerate. Store protein standard solution in refrigerator (2-8°C). The solution is stable for at least 3 months when stored refrigerated.

Procedure:

1. Prepare protein standards (50-400 µg/ml) by diluting the 400 µg/ml protein standard solution in water to a volume of 1 ml in appropriately labelled tubes.

2. **For Blank:** Add 1 ml of water.

3. **For Samples:** Dilute 10 ul of RBC in 10 ml of water. Take 300 ul from this dilution and make up to 1 ml in test tube.

4. Add 1 ml of Lowry reagent solution to standards, blank and samples. Allow solution to stand for 20 minutes at room temperature.

5. With, rapid and immediate mixing add 500 ul of Folin and Ciocalteu's phenol reagent to each tube. Allow to stand for 30 minutes for development of color.

6. Read the absorbance at 750 nm within 30 minutes. Milton Roy (spectronic 3000 array) spectrophotometer was used for reading the absorbance.

7. Plot the absorbance values of the standards versus their corresponding protein concentrations to prepare a calibration curve.

8. Determine the protein concentration of the sample tube from the calibration curve. Multiply the results by the appropriate dilution factor to obtain the protein concentration in the original sample.

6. Measuring superoxide dismutase activity in erythrocytes:

Method: By Spitz D R and Oberley W L. Current protocols in toxicology-Unit 7.6

Chemicals:

1. Potassium phosphate (monobasic)
2. Potassium phosphate (dibasic)
3. Xanthine 1.18 Mmol
4. Nitroblue tetrazolium 2.24 Mmol
5. Diethylenetriaminepantaacetic acid 1.34 Mmol (DETAPAC)
6. Catalase 40 U/ml
7. Bovine serum albumin
8. Bathocuproine disulphonic acid (BCS)

9. Xanthine oxidase XO (0.055 units/ml)

10. Copper Zinc superoxide dismutase (5000 ng/ml)

Reagents:

1. 0.05 M potassium phosphate buffer, pH 7.8 (good for 1 year if refrigerated):
   (a) Solution A: 1 M potassium phosphate, monobasic 136 gm/L.
   (b) Solution B: 1 M potassium phosphate, dibasic, 174 gm/L.
   (c) Solution C Monobasic: 10 ml of solution A diluted to 200 ml with water.
   (d) Solution D Dibasic: 50 ml of solution B diluted to 1 liter with water.
   (e) Add solution C to solution D until pH reaches 7.8 stirring constantly at room temperature.
2. Xanthine 1.18 Mmol (good for 7 days when refrigerated): 0.0045 gm/25 ml. Stir while heating to dissolve.
3. Nitroblue Tetrazolium (NBT) 2.24 Mmol (good for 1 year at 4°C): 0.1832 gm/100 ml, stir to dissolve. Keep in brown bottle.
4. Diethylenetriaminepantaacetic acid (DETAPAC) 1.34 Mmol (good for 1 year at 4°C): 0.2635 gm in 500 ml of phosphate buffer.
5. Catalase 40 U/ml (good for 1 month when refrigerated as 4000 U/ml solution): Take 25 ul of 4000 U/ml and make up to 2.5 ml in phosphate buffer.
6. Bovine Serum Albumin (BSA) (prepare fresh every 3 days): 0.0202 gm/100 ml DETAPAC buffer, store refrigerated.
7. Bathocuproine Disulphonic Acid (BCS), disodium salt hydrate (prepare fresh every 2 days): 0.00565 gm in 1 ml of phosphate buffer, store refrigerated.

8. Xanthine Oxidase (XO) (0.055 U/ml) (prepare fresh daily, keep on ice throughout assay): Prepare stock of 0.2 U/ml, and dilute to 0.055 U/ml to get absorbance in range of 0.017-0.025/minute (0.020/abs/min is ideal).
9. CuZn SOD Standard (5000 ng/ml) (store at -20°C): Dilute vial contents to 3.2 ml with phosphate buffer to get 5 mg/ml concentration. Take 25 ul of this stock and dilute to 25 ml with phosphate buffer.

Procedure:

1. Prepare CuZnSOD standard: 5000 ng/ml solution stored at -20°C until used.
2. Mix stock solutions in the following proportions and volumes to make final assay solution to add to each sample or blank.
3. Final Assay Solution:

Stock solution	Final conc.	Total vol. added (ml)
0.05 M phosphate buffer with 1.34 Mmol DETAPAC and 0.202 mg/ml BSA.	0.05M phosphate buffer 1 Mmol DETAPAC 0.13 mg/ml BSA.	12.9
Catalase 40 U/ml	1.0 units	0.50
NBT 2.24 M	5.6×10^{-5}M	0.50
Xanthine 1.18 Mmol	10^{-4}M	1.70
0.05 M phosphate buffer	0.05M	0.30
BCS 0.01 M	50.0 μM	0.10
	TOTAL	16

4. Adjust blank rate by testing xanthine oxidase. Blank rate should be ideally 0.022 abs/min (add more XO if lower, dilute XO if too high; acceptable range 0.017-

0.025 abs/min. Run reference before testing XO; add 800 ul FAS + 100 ul DETAPAC buffer + 100 ul phosphate buffer for reference.

5. Make sample or standard dilutions such that desired amount of protein can be added in a 100 ul final volume of 0.05 M potassium phosphate buffer. Prepare standards in following order:

SOD conc. (ng)	CuZn standard (ul)	Buffer (ul)
0 blank 1	0	100
2	2	498
4	4	496
6	6	494
8	8	492
10	10	490
25	25	475
50	50	450
500	100	0
0 blank 2	0	100

6. Add mixed FAS 800 ul to each sample, standard or blank.
7. Add xanthine oxidase to the final assay solution containing sample, finger vortex, transfer to cuvette, wait for 1 minute, then monitor absorbance at 560 nm every 15 seconds for 4 minutes. Run two blanks in beginning and at the end, with every set of samples or standards.

8. Spectrophotometer ultrospec 4000-Pharmacia Biotech was used for the kinetic assay.

Result:

1. % inhibition= $\frac{\text{rate of blank-rate of sample}}{\text{rate of blank}} \times 100$

2. One unit of activity is defined as that amount of protein that will inhibit NBT reduction by 50%. Usually one unit of activity is 10-20 μg of protein for total SOD activity.

3. Units of total SOD activity/mg protein can be calculated for a given sample by taking 1000/μg protein per unit.

7. Measuring plasma hydroperoxides:

Method: By Nourooz-Zadeh J *et al.*, 1994.

Chemicals:

1. Ammonium ferrous sulfate
2. Xylenol orange
3. Sulphuric acid (H_2SO_4)
4. Butylated hydroxy toluene (BHT)
5. Methanol (HPLC grade)
6. Triphenylphosphine (TPP)
7. Hydroperoxides 36%

Reagents:

1. Solution A: Dissolve ammonium ferrous sulfate (98.03 mg) in 100 ml of 250 Mmol sulphuric acid. Add xylenol orange 76.06 mg to ammonium ferrous

sulphate solution and the mixture is kept under stirring for about 10 minutes at room temperature.

2. Solution B: Dissolve 969.76 mg BHT in 900 ml methanol.
3. FOX 2 reagent: Mix 1 volume of solution A with 9 volume of B (1A:9B).

Procedure:

1. Prepare 1000 µM hydrogen peroxide stock solution.
2. Prepare dilutions as under to get concentration of 0.5 µM, 0.75 µM, 1 µM, 2 µM, 4 µM, and 8 µM in standard assay:

Conc. (µM)	From	Take (ul)	Add D.W (ul)	Final vol. (ul)
5.5	1000 µM	5.5	994.5	1000
8.33	1000 µM	8.33	991.67	1000
11.11	1000 µM	11.11	988.89	1000
22.22	1000 µM	22.22	977.78	1000
44.44	1000 µM	44.44	955.56	1000
88.88	1000 µM	88.88	911.12	1000

3. **For Standards**: From each of the above standard solution take 90 ul and add 10 ul

   of methanol. Vortex. Incubate at room temperature for 30 minutes.
4. Add 900 ul of FOX2 Reagent. Vortex. Incubate at room temperature for 30 minutes. Centrifuge at 9500rpm for 10 minutes.
5. Take 200 ul for the plate reading (Spectramax 340). Read at 560 nm.

6. **For Samples**: Take 90 ul of plasma into two centrifuge tubes per sample, add 10 ul of methanol to one tube and add 10 ul TPP to other tube. Vortex. Incubate at room temperature for 30 minutes.
7. Add 900 ul of FOX2 reagent. Vortex. Incubate at room temperature for 30 minutes. Centrifuge at 9500rpm for 10 minutes.
8. Take 200 ul for plate reading. Read at 560 nm.
9. **For Blank**: Take 90 ul of double distilled water and add 10 ul of methanol. Vortex. Incubate at room temperature for 30 minutes.
10. Add 900 ul FOX2 reagent. Vortex. Incubate at room temperature for 30 minutes. Centrifuge at 9500rpm for 10 minutes.
11. Take 200 ul for plate reading. Read at 560 nm.

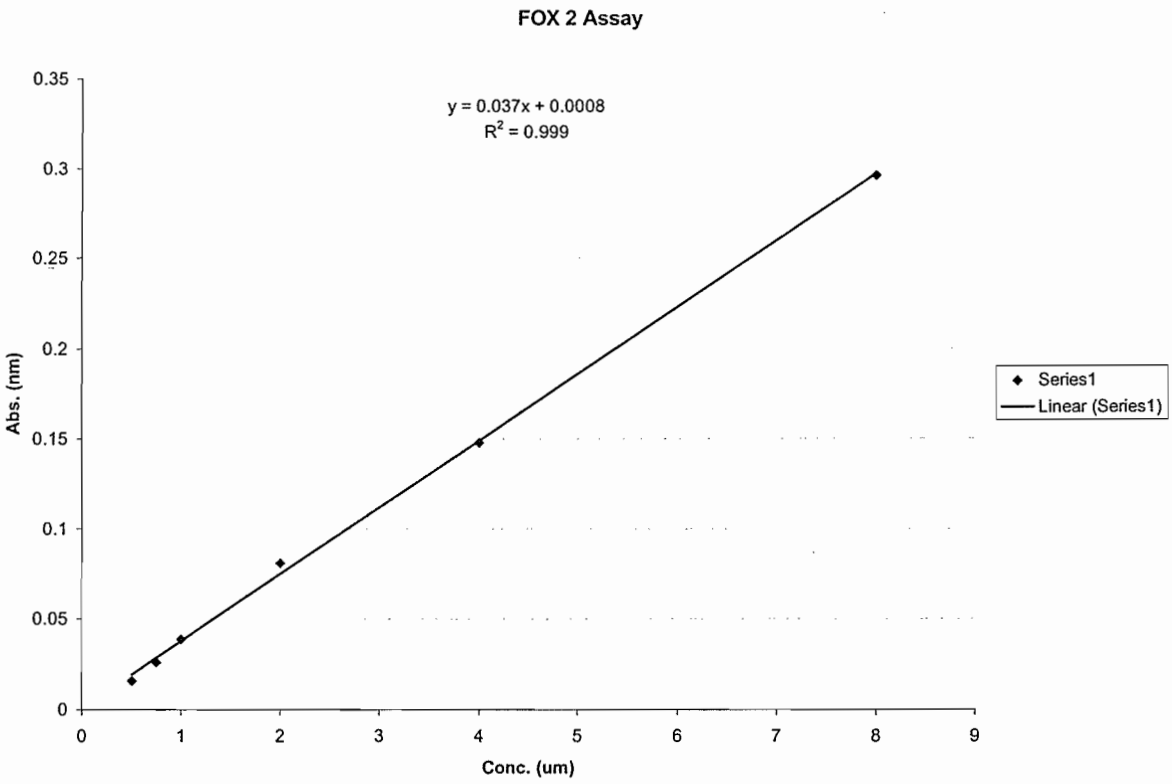


Figure 32. Linear curve of hydroperoxide standards.

Result:

The blank is subtracted by the microplatereader after reading the samples. The ROOH content in the plasma samples is determined as a function of the mean absorbance difference of samples with and without elimination of ROOH's by TPP. Absorbance differences pre and post treatment with TPP range from 0.002 to 0.05.

8. Measuring 8-isoprostanes in urine:

Method: Cayman chemicals Catalog No.516351.

Equipment and Chemicals:

1. A plate reader (spectramax 340) with 405-420 nm filter
2. An adjustable pipettor
3. A source of ultrapure water
4. Test tubes
5. Orbital shaker
6. Vortex mixer
7. Microcentrifuge tubes
8. 1 ml syringe
9. Ethanol (90%)

Reagents:

1. Buffer Preparation:

a) EIA buffer preparation

Dilute the contents of one vial of EIA buffer concentrate (vial#4) with 90 ml of ultrapure water. Rinse the vial completely to remove any precipitates.

b) wash buffer preparation

Dilute 5 ml of wash buffer concentrate (vial#5) to a total volume of 2 liter with ultra pure water. Then add 1 ml of Tween 20 (vial#5a) and mix (use a syringe to measure Tween 20).

2. Standard preparation:

Equilibrate a pipette tip in ethanol by repeatedly filling and expelling the tip with ethanol several times. Using this tip, transfer 100 ul of 8-isoprostane standard (vial#3) into a clean test tube, then dilute with 900 ul of ultra pure water. The concentration of this bulk standard will be 5 ng/ml. Obtain 8 clean test tubes and number them1 to 8. Aliquot 900 ul EIA buffer to tube#1 and 500 ul EIA buffer to tubes #2 to #8.Transfer 100 ul of the bulk standard to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 ul from tube#1 and placing in tube#2; mix thoroughly. Next, remove 500 ul from tube#2 and place it into tube#3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standard should not be stored for more than 24 hours.

Reconstitution of 8-isoprostane AChE tracer

Reconstitute the 100 dtn (1vial) 8-isoprostane tracer (vial#2) with 6 ml EIA buffer. Store at 4°C and use within 4 weeks.

Reconstitution of 8-isoprostane antiserum

Reconstitute the 100 dtn 8-isoprostane Antiserum (vial#1) with 6 ml EIA buffer. Store at 4°C and use within 4 weeks.

3. Sample preparation:

Centrifuge the urine samples at 10000g for 3-5 minutes to remove any precipitate. Dilute the sample with EIA buffer 1:1 and 1:2. The dilution factor could be different for

different samples. If the two different dilutions show good correlation (differ by 20% or less) purification is not required. If you do not see good correlation of the different dilutions, purification is advised.

Plate set up

Each plate (96 wells) must contain at least two blanks (BLK), two non-specific binding wells (NSB), two maximum binding wells (Bo) and eight point standard curve run in duplicate.

Pipetting hints

1. Before pipetting each reagent, equilibrate the pipette tip in that reagent several times.

2. Do not expose the pipette tip to the reagent(s) already in the well.

4. Procedure:

1. Add 100 ul EIA buffer to NSB wells.

2. Add 50 ul EIA buffer to Bo wells.

3. **Isoprostane standards:** Add 50 ul from tube#8 to both of the lowest standard wells (S8). Add 50 ul from tube#7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

4. Add 50 ul of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate.

5. Add 50 ul of 8-isoprostane AChE Tracer to each well **except** the total activity (TA) and the blank (BLK) wells.

6. Add 50 ul of 8-isoprostane Antiserum to each well **except** the TA, the non-specific binding (NSB), and BLK wells.

7. Incubate the plate. Cover each plate with plastic film (item#7) and incubate at room temperature for 18 hours.

8. Develop the plate. When ready to develop the plate, reconstitute one 100 dtn of Ellman's reagent with 20 ml of ultrapure water. Empty the wells and rinse 5 times with wash buffer. Add 200 ul of Ellman's reagent to each well and 5 ul of tracer to total activity wells. Cover the plate with plastic film (Ellman's reagent is unstable and should be used in one day; protect it from light when not in use). Put the plate on an orbital shaker and allow the plate to develop in the dark for 60-90 minutes.

9. Read the plate at 412 nm. Before reading each plate, wipe the bottom of the plate with a clean tissue to remove finger prints, dirt etc. Be certain that Ellman's reagent has not splashed up on the plate cover. The plate may be checked periodically until the Bo wells have reached a minimum of 0.3 absorbance units (A.U) (blank subtracted). Read the plate when the absorbance of the Bo wells is in the range of 0.3-0.8 A.U. If the absorbance of the wells exceeds 1.5, wash the plate, add fresh Ellman's reagent and let it develop again.

10. Calculate the result using the Cayman Chemical spread sheet.

9. Questionnaire: (From Dr. Taylor's Study)

DIET AND DIABETES RESEARCH STUDY

This information will be kept strictly confidential.

1. **Name** _____

   Address _____

   Telephone number(s): Home_____ Work_____Cell number_____

   Email address: _____

   Age: ____years

   Sex: ___male or ___female

2. **Are you allergic or intolerant to any food products [e.g. flax, eggs, wheat flour (gluten), or yogurt?** Yes ___ No ____

   If yes, please list them:_____

3. **Please answer the following questions about your Type 2 diabetes.**

   a. When were you first diagnosed with diabetes?

   _____

   b. Do you attend a Diabetes Education Centre? If yes, which one?

   _____

   c. Who is your physician?

   _____

   d. What is your physician's clinic name or clinic address and/or phone number?

   _____

   e. Do you control your diabetes with

   Oral glycemic agents (e.g. glyburide, metformin, Acrabose)?

   Yes____ No____

   If yes, please specify what you take: _____

   Injecting insulin? Yes____ No____

4. **Do you have any of the following medical conditions?**

__Oral or gum disease

__Hypertension (high blood pressure)

__Hypercholesterolemia (high blood cholesterol)

__Hyperlipidemia (high blood lipids)

__Heart Disease

__Heart Attack

__Stroke

__Kidney Disease

__Gastrointestinal Disease

__Liver Disease

__Recent surgery

__Other: _____

5. **Do you take any medications or prescription drugs?**

Yes ___ No ____

If yes, please list them:_____

6. **Do you eat foods containing flax?** If yes, what type of foods and how often do you eat each food product?

_____

7. **Do you eat foods or consume supplements containing soy or soy isoflavones?** If yes, what type of foods and/or supplements and how often do you consume them?

_____

8. **Do you take fish oil or flaxseed oil capsules?** If yes, how often and what amount do you take?

_____

9. **Do you smoke?** If yes, how many cigarettes (or other tobacco), for example, per day, per week, or per month?

_____

10. **Do you consume alcohol?** If yes, how many drinks, for example, per day, per week, or per month?

_____

11. **On average, how many meals per week do you eat at restaurants or at someone else's home?**

__0-4

__5-10

__11 or more

12. **Do you have any plans to travel outside of Winnipeg during the duration of the study?**

Yes ___ No ____

If yes, will this affect your participation in the study and the consumption of the food products?

_____

13. **Do you control your diabetes with diet, exercise, or other lifestyle choices?**

Yes____ No____ If yes, please explain how:

14. **Do you monitor your blood sugar levels?** Yes____ No____

a) If yes, how do you monitor, and how often do you monitor?

b) If blood sugars are too high, do you take steps to prevent it from happening in the future? (for example, diet changes, exercise, drinking water, etc.)

TABLE 1 – Anthropometric measurements. Control (n=10); Milled flax (n=13); Flax Oil (n=12). Values are expressed as means ± SE.

Group	Weight (Kg)	BMI	Waist (Cm)
Control			
0 wk	92.83 ± 1.72	33.41 ± 0.43	108.33 ± 1.27
4 wk	96.25 ± 1.98	34.63 ± 0.54	109.16 ± 1.40
8 wk	97.42 ± 2.02	35.04 ± 0.55	108.21 ± 1.34
12 wk	95.53 ± 2.05	34.35 ± 0.57	107.95 ± 1.25
Milled Flax			
0 wk	88.41 ± 1.42	31.56 ± 0.40	105.51 ± 0.91
4 wk	89.22 ± 1.43	31.87 ± 0.41	102.60 ± 1.05
8 wk	88.66 ± 1.41	31.71 ± 0.43	101.80 ± 1.03
12 wk	88.58 ± 1.39	31.67 ± 0.41	101.41 ± 1.09
Flax Oil			
0 wk	93.29 ± 1.55	32.71 ± 0.66	104.92 ± 1.11
4 wk	94.81 ± 1.53	33.20 ± 0.64	104.78 ± 1.04
8 wk	94.40 ± 1.49	33.06 ± 0.63	104.78 ± 1.16
12 wk	94.26 ± 1.48	33.02 ± 0.63	103.09 ± 1.04

* This data is obtained from Dr. Taylor's study.

TABLE 2 – Fasting glucose and glycated haemoglobin. Values are means ± SE for the flaxseed (n=13), flaxseed oil (n=12) and control (n=10) groups.

Group	Glucose (Mmol/L)	Glycated Hb (%)
Milled Flax		
0 wk	6.6 ± 0.1	6.3 ± 0.1
4 wk	6.6 ± 0.1	6.3 ± 0.1
8 wk	6.6 ± 0.1	6.4 ± 0.1
12 wk	6.4 ± 0.1	6.3 ± 0.1
Flax Oil		
0 wk	6.9 ± 0.1	6.5 ± 0.1
4 wk	7.3 ± 0.1	6.6 ± 0.1
8 wk	6.9 ± 0.1	6.8 ± 0.1
12 wk	6.8 ± 0.1	6.8 ± 0.1
Control		
0 wk	8.6 ± 0.3	7.6 ± 0.2
4 wk	8.2 ± 0.3	7.7 ± 0.6
8 wk	8.2 ± 0.3	7.8 ± 0.2
12 wk	7.9 ± 0.3	7.7 ± 0.2

* This data is obtained from Dr. Taylor's study.

TABLE 3 – Fasting lipid profile. Values are means ± SE for the flaxseed (n=13), flaxseed oil (n=12) and control (n=10) groups.

Group	Cholesterol	TG	HDL	LDL	Total Chol/HDL	LDL/HDL
Milled Flax						
0 wk	4.9 ± 0.1	1.9 ± 0.1	1.3 ± 0.0	2.7 ± 0.1	4.0 ± 0.1	2.2 ± 0.1
4 wk	4.7 ± 0.1	1.7 ± 0.1	1.2 ± 0.0	2.6 ± 0.1	3.8 ± 0.1	2.2 ± 0.1
8 wk	4.8 ± 0.1	1.8 ± 0.1	1.2 ± 0.0	2.7 ± 0.1	4.0 ± 0.1	2.3 ± 0.1
12 wk	4.6 ± 0.0	1.8 ± 0.1	1.2 ± 0.0	2.6 ± 0.0	4.0 ± 0.1	2.2 ± 0.1
Flax Oil						
0 wk	5.1 ± 0.1	2.2 ± 0.1	1.2 ± 0.0	2.9 ± 0.1	4.3 ± 0.1	2.5 ± 0.1
4 wk	5.3 ± 0.1	2.6 ± 0.2	1.2 ± 0.0	3.0 ± 0.1	4.5 ± 0.2	2.7 ± 0.1
8 wk	4.7 ± 0.1	2.1 ± 0.1	1.1 ± 0.0	2.7 ± 0.1	4.3 ± 0.2	2.4 ± 0.1
12 wk	5.0 ± 0.1	2.3 ± 0.2	1.1 ± 0.0	2.9 ± 0.1	4.5 ± 0.1	2.7 ± 0.1
Control						
0 wk	5.4 ± 0.1	1.6 ± 0.1	1.3 ± 0.0	3.4 ± 0.1	4.4 ± 0.2	2.8 ± 0.1
4 wk	5.3 ± 0.4	1.5 ± 0.1	1.3 ± 0.1	3.2 ± 0.2	4.2 ± 0.3	2.6 ± 0.2
8 wk	5.1 ± 0.2	1.8 ± 0.1	1.3 ± 0.0	3.0 ± 0.1	4.2 ± 0.1	2.5 ± 0.1
12 wk	5.0 ± 0.2	1.5 ± 0.1	1.3 ± 0.0	3.0 ± 0.1	4.2 ± 0.1	2.6 ± 0.1

* This data is obtained from Dr. Taylor's study.

TABLE 4 – Plasma Vitamins E, C and A. Values are expressed as mean ± SE for the milled flaxseed (n=13), flaxseed oil (n=12) and control (n=10) groups.

Group	Vitamin E (µg/ml)	Vitamin C (mg/dl)	Vitamin A (µg/ml)
Control			
0 wk	15.00 ± 2.33	0.71 ± 0.14	1.51 ± 0.14
4 wk	13.29 ± 1.96	0.55 ± 0.17	1.40 ± 0.12
8 wk	12.02 ± 1.68	0.68 ± 0.16	1.41 ± 0.11
12 wk	12.51 ± 1.28	0.51 ± 0.16	1.42 ± 0.10
Milled Flax			
0 wk	12.69 ± 1.31	0.94 ± 0.14	1.87 ± 0.28
4 wk	12.57 ± 1.08	0.69 ± 0.12	1.50 ± 0.12
8 wk	16.03 ± 1.70	0.60 ± 0.10	2.05 ± 0.21
12 wk	7.11 ± 1.37	0.89 ± 0.12	1.43 ± 0.09
Flax Oil			
0 wk	11.8 ± 0.83	0.52 ± 0.11	1.45 ± 0.12
4 wk	10.13 ± 0.75	0.19 ± 0.06	1.42 ± 0.15
8 wk	12.06 ± 0.61	0.27 ± 0.05	1.73 ± 0.13
12 wk	10.39 ± 0.95	0.47 ± 0.08	1.59 ± 0.22

TABLE 5 – Erythrocyte SOD, CAT activity and plasma total antioxidant property. Values expressed as mean ± SE for the milled flaxseed (n=13), flaxseed oil (n=12) and control (n=10) groups.

Groups	SOD (U/mg pr.)	CAT (U/mg pr.)	Total Antioxidant Property (μM)
Control			
0 wk	33.00 ± 3.69	77.20 ± 6.04	1037.00 ± 68.82
4 wk	93.70 ± 16.81	206.10 ± 36.03	981.00 ± 69.99
8 wk	58.80 ± 5.08	153.90 ± 18.27	1080.40 ± 61.68
12 wk	65.60 ± 4.56	154.10 ± 13.24	1046.40 ± 82.55
Milled Flax			
0 wk	76.76 ± 8.55	230.23 ± 23.60	977.33 ± 41.31
4 wk	66.46 ± 8.39	180.61 ± 25.63	963.95 ± 45.95
8 wk	81.07 ± 9.19	170.00 ± 11.28	958.08 ± 54.71
12 wk	69.30 ± 7.57	167.76 ± 23.11	952.70 ± 47.08
Flax Oil			
0 wk	45.66 ± 2.77	138.58 ± 11.03	1056.89 ± 47.27
4 wk	83.91 ± 10.13	221.50 ± 21.22	1083.49 ± 78.05
8 wk	62.58 ± 3.28	179.91 ± 12.84	1029.37 ± 59.77
12 wk	67.75 ± 9.38	168.08 ± 11.59	1074.17 ± 57.64

TABLE 6 – Urinary isoprostanes, plasma hydroperoxides and plasma protein carbonyl. Values expressed as mean ± SE for the milled flaxseed (n=13), flaxseed oil (n=12) and control (n=10) groups.

Group	Isoprostanes (pg/ml)	Hydroperoxides (μM)	*Protein Carbonyl (nmol/mg pr.)
Control			
0 wk	1567.00 ± 338.14	0.76 ± 0.10	0.33 ± 0.10
4 wk	1115.80 ± 353.62	0.57 ± 0.10	N/A
8 wk	956.30 ± 228.75	0.48 ± 0.08	N/A
12 wk	1455.20 ± 371.01	0.56 ± 0.05	0.25 ± 0.09
Milled Flax			
0 wk	954.66 ±189.40	0.65 ± 0.11	0.27 ± 0.12
4 wk	741.23 ± 141.52	0.47 ± 0.07	N/A
8 wk	752.23 ± 147.78	0.49 ± 0.04	N/A
12 wk	784.53 ± 200.41	0.46 ± 0.04	0.40 ± 0.17
Flax Oil			
0 wk	583.91 ± 118.96	0.57 ± 0.06	0.42 ± 0.36
4 wk	477.75 ± 66.82	0.33 ± 0.05	N/A
8 wk	624.00 ± 123.93	0.48 ± 0.05	N/A
12 wk	492.91 ± 70.19	0.47 ± 0.06	0.28 ± 0.12

* Data provided by Kevin Cockell, Health Canada, Ottawa.
* Urinary creatinine data was obtained from Dr. Taylor's study to express urinary isoprostanes as pg/mg creatinine.

TABLE 7: Main effects for each of the biochemical parameters measured.

	P >F		
Parameter	**Time Effect**	**Group Effect**	**Group x Time**
Vitamin C (mg/dl)	0.0002	0.0183	0.0314
Vitamin E (µg/ml)	0.0014	**NS**	**NS**
Vitamin A (µg/ml)	0.0134	**NS**	**NS**
SOD (U/mg protein)	0.0010	**NS**	0.0078
CAT (U/mg protein)	0.0214	0.0100	<0.0001
Total Antioxidant Property (µM/L)	0.0014	**NS**	**NS**
Isoprostanes (pg/mg Cr)	0.0105	0.0002	**NS**
Hydroperoxides (µM/L)	0.0014	**NS**	**NS**

* Group x Time = Interaction between group and time