LIPID PEROXIDATION AND ANTIOXIDANT STATUS IN TYPE 2 DIABETES MELLITUS IN GHANA.

NGISSAH PATRICK

INDEX NUMBER: 10363405

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MPHIL CHEMICAL PATHOLOGY DEGREE.

JULY, 2013

DECLARATION

This thesis is submitted to University of Ghana, School of Graduate studies through the College of Health Sciences, Medical School, and Department of Chemical Pathology. I hereby declare that with the exception of references to other people's work, which have been duly acknowledged, this thesis is the outcome of my own research conducted at the Department of Biochemistry and Centre of (UGMS, Korle-Bu) under the supervision of Dr. Asare-Anane Henry and Dr. Amanquah Seth Dortey. This thesis presents results of original research undertaken by me and neither all nor parts of this thesis have been presented for another degree.

……. Date: ……

NGISSAH PATRICK

(Student)

…… Date:……..

DR. HENRY ASARE-ANANE

(Supervisor)

……. Date: ……

DR. AMANQUAH SETH DORTEY

(Supervisor)

DEDICATION

All glory and honour be unto God most high for His loving-kindness and faithfulness. I dedicate this work to my mum, who has supported me to attain greater heights in academia and my lovely brother Ngissah Melchizedek.

ACKNOWLEDGEMENT

I take this opportunity to thank everyone who has helped me during the course of my study and in preparing this dissertation.

First and foremost I thank my God for His bountiful grace and being the provider and the strength of my life. My Father in heaven I am grateful to you.

I wish to express my sincere thanks to Dr. Sylvester Yaw Oppong, Head of Department of Chemical Pathology, UGMS, Korle-Bu, for giving me this opportunity and being a constant source of inspiration.

It is a great honour to express my sincere and warm gratitude to Dr. Asare-Anane Henry, Department of Chemical Pathology, for his overwhelming encouragement, unflinching support and timely help, whenever needed.

Guidance in the proper direction is a necessity for any form of success in life. It gives me immense pleasure to express my deepest gratitude to my teacher and guild Dr. Amanquah Seth Dortey, Department of Chemical Pathology for his genuine interest, sincere evaluation and timely help in the making of this dissertation.

I am indebted to my mother, Ernestina Ebbah for supporting financial towards my study. God bless you mother.

I extend my thanks to technical and non-technical staff of the department of Biochemistry.

My thanks to all members of the Department of Chemical Pathology.

To my colleagues, Afua, Priscilla and Collins, I say God bless you for the wonderful time we have had together.

ABSTRACT

An imbalance between reactive oxygen species production and antioxidant scavenging has been implicated in type 2 diabetes mellitus. Reports indicate that several complications of diabetes mellitus result from increased activity of free radicals and accumulation of lipid peroxidation products leading to oxidative stress. The study was aimed at evaluating lipid peroxidation and antioxidant status along with lipid profile of type 2 diabetes mellitus patients and age- matched healthy subjects. One hundred subjects of type 2 diabetes mellitus without any complications (mean age; 50.25± 5.57 years) and one hundred healthy subjects (mean age; 48.35 ± 6.26 years) were included in this study. Body Mass Index (BMI), systolic and diastolic blood pressure were measured. Fasting blood sample was collected for the analysis of fasting plasma glucose, glycated hemoglobin, serum malondialdehyde, and activities of superoxide dismutase and glutathione peroxidase, and serum lipid profile in both the groups. Compared with control subjects, diabetic subjects had significantly higher Total cholesterol (6.76±1.11 vs 0.94±0.23 mmol/L), triglyceride (1.29±0.34 vs 0.94±0.23 mmol/L), LDL-cholesterol (3.96 ± 1.47 vs 1.46±0.69 mmol/L), serum malondialdehyde significantly increased (4.40±1.96 vs 2.75±1.05 µM) whereas, HDL- cholesterol (1.74±0.46 vs1.46±0.69 mmol/L), and activities of superoxide dismutase (3.80± 1.64 vs 10.39±2.55 U/Ml) and Glutathione peroxidase (129.96±16.75 U/L) were significantly reduced in DM type-2 patients as compared to controls ($p < 0.05$). BMI was significantly higher in type 2 diabetic subjects compared to healthy control subjects ($p < 0.05$). Oxidative stress is raised in type 2 DM patients. The risk factors in the development of complications associated with type 2 diabetes mellitus include increased oxidative stress, deranged lipid profile and decreased antioxidant status of patients. Future studies need to focus on gathering large sample sizes to clarify the relationship between antioxidants depletion and type 2 diabetes mellitus.

LIST OF TABLES

Table 4.10 Anthropometric characteristics of the study subjects……..……51

Table 4.11 Anthropometric characteristics between males and females type 2 diabetic subjects…….…..52

Table 4.12 Differences in anthropometric characteristics between males and females in control subjects……..53

Table 4.13 Biochemical characteristics of type 2 diabetic and control subjects……...54

Table 4.14 Biochemical characteristics between males and females control subjects…55

Table 4.15 Biochemical characteristics between males and females in diabeticsubjects……..……....56

Table 4.16 Oxidative stress indices of the study subjects……..57

Table 4.17 Oxidative stress indices between males and females in control subjects…….....57

Table 4.18 Oxidative stress indices between men and women in diabetic subjects……...…….……...58

Table 4.19 Pearson's correlation table of anthropometric variables in the type 2 diabetic subjects……...59

Table 4.20 Pearson's correlation table of anthropometric and biochemical variables in the type 2 diabetic subjects….…….60

Table 4.21 Pearson's correlation table of anthropometric variables and oxidative stress indices in the type 2 diabetic subjects……...…….61

Table 4.22 Pearson's correlation table between biochemical variables in the type 2 diabetic subjects……..……..…….62

Table 4.23 Pearson's correlation table of biochemical variables and oxidative stress indices in the type 2 diabetic subjects……..……..63

Table 4.24 Pearson's correlation table of anthropometric and biochemical variables in the type 2 diabetic subjects…….…….....64

TABLE OF CONTENTS

PAGES

DECLARATION……I

DEDICATION……...II

ACKNOWLEDGEMENT…… ….……III

ABSTRACT……..…….…..IV

TABLE OF CONTENTS.....VI

LIST OF ABBREVIATIONS ……..…XIV

CHAPTER ONE……....1

1.0 INTRODUCTION ……...……...**1**

1.1Background…….....1

1.2 Study hypothesis……....6

1.3Problem Statement…….….6

1.4 Justification…….…...…6

1.5Aim…….7

1.6 Specific Objectives.....7

CHAPTER TWO.....…...**8**

2.0 Literature Review ……..……8

2.10 Definition of diabetes mellitus……..……8

2.1.1 Types of diabetes mellitus…….……8

2.1.2 Diagnosis of diabetes mellitus……...9

2.1.3 Pathogenesis of type 2 diabetes mellitus…….……..…….9

2.1.4Aetiological factors underlying of diabetes mellitus…….11

2.1.5 Complication of diabetes mellitus…….…...11

2.1.6 Overview of Type 2 DM and Oxidative stress…….12

2.1.7 Free radicals…….……..13

2.1.8 Reactive Oxygen Species…….....14

2.2.0 Mechanism of production of reactive oxygen species……..…16

2.2.1 Endogenous production of reactive oxygen species ……16

2.2.2 Exogenous production of reactive oxygen species ……..…16

2.2.3.0 Types of reactive oxygen species……..17

2.2.3.1Superoxide anion (O_2^-)……...…….17

2.2.3.2 Hydroxyl radical……....17

2.2.3.3 Hydrogen peroxide……..…...18

2.2.3.4 Singlet…….……...19

2.2.3.5 Peroxyl radical……..19

2.2.4.0Targets of reactive oxygen species……20

2.3.0 Antioxidants …….....21

2.3.1.0 Classification of Antioxidants……22

2.3.1.1 Naturally-occurring enzymatic antioxidants…….…….....22

2.3.1.2 Preventive antioxidants……..……....23

2.3.1.3 Scavenging or chain-breaking antioxidants……...……...…….23

2.4.0 Overview of Oxidative stress…… …….……...…23

2.4.1.0Sources of Oxidative stress in diabetes mellitus…….25

2.4.1.1 Auto-oxidative Glycosylation……...…....25

2.4.1.2 Auto-oxidative of glucose and Amadori product.....……..25

2.4.1.3 Antioxidant status……..27

2.5.0 Lipid peroxidation…… ……...……...28

2.5.1 Lipid peroxidation in diabetes…….…….29

2.5.2 Mechanism of antioxidant action on lipid peroxidation…….…..30

2.6.0 Biochemical markers of free radical mediated oxidative stress…….....31

2.6.1 Superoxide dismutase…….…….....31

2.6.2 Malondialdehyde (MDA)……...……..….32

2.6.3Glycated hemoglobin (Hb_{A1C})…….....33

2.6.4 Glutathione Peroxidase (GPx)……..34

CHAPTER THREE ……..……...36

3.0 METHODOLOGY……..……36

3.1.0 Study design……..36

3.2.0 Inclusion criteria…….....36

3.3.0 Exclusion criteria……...……...37

3.4.0. Minimum Sample size determination……...……...37

MATERIALS…….38

3.5.0 Anthropometric variables……..39

3.6.0 Sample preparation ……...……...39

3.7.0 Metabolic biochemical markers investigation……...……...39

3.7.1 Estimation of blood glucose…….……39

3.7.2 Estimation of Glycosylated haemoglobin (Hb_{A1C}) ……..41

3.7.3 Estimation of Total cholesterol ……43

3.7.4 High density lipoprotein (HDL) cholesterol ……...…….....43

3.7.5 Triglycerides…….44

3.7.6 Low density lipoprotein (LDL) cholesterol …….45

3.8.0 Oxidative stress indices……..…45

3.8.1 Malondialdehyde (MDA)……...…45

3.8.2 Glutathione peroxidase (GPx)……...….47

3.8.3 Superoxide dismutase (SOD)……49

3.9.0 Statistical Analysis……50

3.10.0 Expected outcome……...50

3.11.0 Dissemination of the study outcome……...50

CHAPTER FOUR……51

4.0 Results……...…51

4.10 Anthropometric parameters in the subject population……...……51

4.11 Anthropometric characteristics between male and female in type 2 diabetic subjects……...….52

4.12 Anthropometric characteristics between male and female in control subjects…..….52

4.13 Biochemical Parameters of the study populations……..…53

4.14 Biochemical characteristics of males and females in the control subjects……....54

4.15 Biochemical characteristics of males and females in the diabetic subjects…….....55

4.16. Oxidative stress indices of the study subjects……...…56

4.17. Oxidative stress indices between males and females in control subjects…..…...…57

4.18. Oxidative stress indices between males and females in diabetic subjects……58

4.19 Associations between anthropometric variables in type 2 diabetic subjects…….58

4.20 Associations between anthropometric and biochemical variables in type 2 diabetic subjects……...59

4.21 Association between anthropometric variables and oxidative stress indices in type 2 diabetic subjects……..…60

4.22. Associations between biochemical variables in type 2 diabetic subjects…….61

4.23 Associations between biochemical variables and oxidative stress indices……62

4.24. Associations between oxidative stress indices in type 2 diabetic subjects……...63

CHAPTER FIVE…….65

5.0 Discussion……...65

Conclusion ……....70

Limitations of the study…….70

Recommendation……...71

Reference……...72

Appendices…….90

Appendix I Consent form of study participants……91

Appendix II questionnaire for the study……....93

LISTS OF ABBREVIATIONS

3, 5-DHBS:	3-5 dichloro-2-hydroxybenzene
4-AAP:	4-aminoantipyrine
ADA:	American Diabetes Association
ADP:	Adenosine 5'-diphosphate
AGE:	Advanced glycation end-products
AIDS:	Acquired Immune Deficiency Syndrome
ALE:	Advanced lipoxidation end products
ANOVA:	Analysis of Variance
ATP:	Adenosine 5'-triphosphate
BMI:	Body mass index
CAT:	Catalase
cGMP:	Cyclic Guanosine monophosphate
CHD:	coronary heart disease
CHER:	Cholesterol esterase
CHOD:	Cholesterol oxidase
CI:	Confidence interval

CML	Carboxymethly-lysine
DAP	Dihydroxyacetone phosphate
DBP	Diastolic blood pressure
DD	Duration of disease
DNA	Deoxyribonucleic acid
DTC	2,4-dinitrophenylhydrazine copper sulphate
ECSOD	Extracelluar superoxide dismutase
EDTA	Ethylene diamine tetra acetic acid
ETC	Electron transport chain
FFA	Free fatty acid
FPG	Fasting plasma glucose
G-3-P	Glycerol-3-phosphate
GFAT	Glutamine fructose-6-phosphate amidotransferase
GK	Glycerol kinase
GLUT4	Glucose transporter 4
GOD-POD	Glucose oxidase- Peroxidase
GPO	Glycerophosphate oxidase

GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
Hb_{A1C}	Glycated hemoglobin
HDL	High density lipoprotein cholesterol
HK	Hexokinase
IFG	Impaired fasting glucose
LDL	Low density lipoprotein cholesterol
LPL	lipoprotein lipase
MDA	Malondialdehyde
NAD+	.Oxidized nicotinamide adenine dinucleotide
NADH	.Reduced nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa B
PI3K	Phosphatidylinositol 3 kinase
NADP+	Nicotinamide adenine dinucleotide phosphate (oxidized form)

NADPH	Reduced Nicotinamide adenine dinucleotide phosphate
NDMRC	Diabetes Management and Research Centre
NRP	Non-radical products
OD	Optical density
PKC	Protein kinase C
POD	Peroxidase
PUFA	Poly unsaturated fatty acids
RAGE	Receptor of advanced-glycation end-products
ROS	Reactive oxygen species
S.BP	Systolic blood pressure
SD	Standard deviation
SOD	Superoxide dismutase
TODB	N,N-Bis(4-sulfobutyl)-3-methylaniline
T2DM	Type 2 diabetes mellitus
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substance
TBHA	2, 4, 6- Tribromo-3-hydroxy-benzoic acid

TCA	Trichloroacetic acid
TCHOL	Total cholesterol
TG	pertriglyceridaemia
TG	Triglycerides
TNF-α	Tumor necrosis factor-alpha
VLDL	Very low density lipoprotein cholesterol
WC	Waist circumference
WHO	…World Health Organization
WST-1	Water-Soluble Tetrazolium salt
XO	xanthine oxidase

CHAPTER ONE

1.0 Introduction

1.1 Background

Diabetes mellitus describes a heterogenous metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances in carbohydrate, fat and protein metabolism, resulting from defects in insulin secretion, insulin action, or both (WHO, 1999). It may be present with characteristics symptoms such as polydipsia, polyuria, and polyphagia and weight loss. Diabetes is one of the common non - communicable disorders worldwide. Diabetes mellitus is classified into Type 1 which refers to a condition where there is chronic progressive beta cell destruction and Type 2 which is the most common type of diabetes. Type 2 diabetes mellitus is due to the decreased biological response to insulin. In the early stages, beta cells respond to the insulin resistance by secreting increased quantities of insulin and thus maintaining blood sugar at normal levels. But gradually the beta cells begin to fail and the insulin levels decrease, resulting in the rising of blood sugar.

In the year 2000, the global number of individuals with diabetes was estimated to be 171 million (2.8% of the world's population), and this figure has been projected to increase to 366 million (6.5%) by 2030, 298 million of whom will be living in developing countries (WHO, 1998). Type 2 diabetes mellitus has reached epidemic proportions with explosive increase in incidence worldwide over the past few decades. Although type 2 diabetes mellitus is more prevalent in developed countries, the increase in incidence seems to be more pronounced especially in populations that are experiencing rapid westernization

(Zimmet *et al.*, 2002).

Although diabetes was thought to be rare in sub-Saharan Africa, recent studies from some countries suggests that the disease may now be more common in sub-Saharan Africa than previously thought (Cooper *et al.*, 1997, Mbanya *et al.*, 1999, Aspray *et al.*, 2000). Though epidemiological data on the prevalence of diabetes in Ghana is scanty, evidence suggests that it is on the increase. In the 1950s, the prevalence of diabetes among an outpatient urban population in Accra was estimated at less than 0.5% (Dodu, 1958). The impression was therefore created among policy makers that diabetes is rare in Ghanaians. However, (Amoah *et al.*, (2002)); reported a high prevalence rate of 6.3%. Also, study on the prevalence and socio-demographic aspects of overweight and obesity among residents from rural and urban Accra showed that overall crude prevalence were 23.4% and 14.1%, respectively (Amoah, 2003).

There is a correlation between chronic hyperglycemia and long-term complications in diabetes. The long-term effects of diabetes include progressive development of microvascular complications, particularly in the eye and the kidney, and an increased frequency of macro vascular disease such as peripheral vascular and coronary heart disease (Benneth, 1989). The microvascular and macrovascular complications are the major cause of morbidity and mortality in patients with diabetes mellitus. However, diabetic patients often die from macrovascular disease and major mortality is the coronary heart disease (CHD) (WHO, 1998). It is possible that long term, high blood glucose levels in uncontrolled diabetes mellitus is the cause of glucose autooxidation,

nonenzymatic protein glycation and activation of polyol pathway with increase oxidative stress (Sozmen *et al.,* 2001 and Aybek *et al.,* 2004).

Oxidative stress is the result of the imbalance in pro-oxidant/antioxidant ratio in favor of the former, potentially leading to macromolecules and cell dysfunction (Nehru *et al.*, 2004 and Su *et al.,* 2008). Enhanced oxidative stress contributes to the deterioration of pancreatic β-cell progressively due to glucose toxicity, which leads to severe impairment of glucose-stimulated insulin secretion, apparent degranulation of β-cells and decreased β-cell numbers, resulting in the pathogenesis of diabetes mellitus (Gorogawa *et al.*, 2002 and Schinner *et al.,* 2005). In normal physiological conditions, there is a balance in the generation of reactive oxygen/nitrogen species (ROS/RNS) and antioxidant defense system to deactivate and protects organisms against ROS/RNS toxicity. In diabetes, impairment in the pro-oxidant/antioxidant balance can damage cellular macromolecules, leading to protein modification and lipid peroxidation.

Lipid peroxidation is an autocatalytic free radical-mediated destructive process whereby poly-unsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides (Freeman *et al.,* 1982 and Slater *et al.,* 1984). By-products of lipid peroxidation such as conjugated dienes and malondialdehyde (MDA) are increased in type 2 diabetes mellitus subjects. MDA is generated as a relatively stable end product from the oxidative degradation of poly-unsaturated fatty acids (PUFA) (Horton *et al*., 1987 and Pasaogu *et al.,* 2004). This free radical-driven lipid peroxidation has been causatively implicated in the aging process (Mamett *et al.,* 2000 and Poon *et al.,* 2004),

atherosclerosis (Cavalca *et al.*, 2001), Alzheimer's disease (Markes berry and Lovell, 1998) and cancer (Niedernhofer *et al*., 2003). Serum MDA has been used as a bio-marker of lipid peroxidation and has served as an indicator of free radical damage.

Hyperlipidemia has also been reported as one of the causative factors for increased lipid peroxidation in diabetes mellitus (Losada and Alio 1996 and Rosen *et al*., 2001). The lipid peroxidation pathways enhance tissue degeneration and cause several complex syndromes in diabetic patients such as cataracts, renal dysfunction, nerve damage and atherosclerosis. Atherosclerosis leads to the coronary heart disease which is the major cause of death among diabetes subjects.

Although the underlying mechanisms of the pathogenesis of type 2 diabetes mellitus still remain to be examined, oxidative stress has been shown to be responsible, at least in part, for the progression of type 2 diabetes mellitus and its complications. This is supported by increased oxidative damage to lipids and impaired antioxidants in these patients (Rosen *et al.*, 2001) , as well as the use of some antioxidant nutrients as adjuvants or dietary supplements in the management of type 2 diabetes mellitus (Bonnefont-Rousset *et al.*,2000 and Peerapatdit *et al.*, 2006). Apart from lipid peroxidation used as a marker for oxidative stress, a variety of natural antioxidants exist to scavenge ROS and prevent oxidative damage to biological membranes.

The term antioxidant has been defined as any substance, exogenous or endogenous in nature, that delays or inhibits oxidative damage to a target molecule (Halliwell and Gutteridge, 2007) and protects biologically important molecules such as DNA, proteins,

and lipids from oxidative damage and consequently reduce the risk of several chronic diseases (Yu, 2001). Common antioxidants include the vitamins A, C, and E, as well as glutathione and the enzymes superoxide dismutase, catalase and glutathione reductase. Other antioxidants include α–lipoic acid, mixed carotenoids, coenzyme Q_{10}, several bioflavonoids, and antioxidant minerals (copper, zinc, manganese, and selenium), and the cofactors (folic acid, vitamins B_1, B_2, B_6, B_{12}). They work in synergy with each other and against different types of free radicals. Hyperglycemia impairs the endogenous antioxidant defense mechanism in many ways during diabetes mellitus (Saxane, 1993 and Martin–Gallan *et al*., 2003) reported that people with diabetes do not have enough antioxidants defenses, but, in contrast, too much of the free radical induced damaged. One group of these antioxidants is intracellular enzymatic action, which include superoxide dismutase (SOD), the enzyme which dismutates superoxide to hydrogen peroxide, and other two hydrogen peroxide-scavenging enzymes that is glutathione peroxidase (GPx) and catalase (CAT), which convert hydrogen peroxide to water. These antioxidant enzymes in blood have been cited as markers for vascular injury in type 2 diabetes mellitus (Kalaivanam *et al.,* 2006 and King, 2008)

In patients with type 2 diabetes mellitus, increased oxidative stress and lower concentrations of antioxidants have been reported but results were inconsistent. MDA level was found to be significantly lower in diabetic subjects with (Griesmacher *et al.*,1995, Sundaram *et al.*, 1996,Collier *et al*.,1992 and Kedziora-Kornatowska *et al*, 1998) and without complications (Dincer *et al.,* 2002 and Ceriello *et al.,* 1997). Others have reported no change in indices of oxidative stress (Nurooz-Zadoh *et al.,* 1996 and Van der Jagt *et al.*, 2001). Data on oxidative stress in patients of type 2 diabetes with and without

complications is scanty especially in Ghana. The study of the oxidative stress status may be the knowledge base for understanding of the pathogenic mechanisms of complications in diabetes and may have important implications regarding antioxidant supplements in order to slow progression, select optimal therapies and prevent plaque complications and their consequences.

1.2 Study hypothesis

Type 2 diabetes mellitus is not associated with increased oxidative stress and concomitant decrease in antioxidant status.

1.3 Problem statement

There is evidence that diabetes leads to depletion of the cellular antioxidant defense system and increased levels of reactive oxygen species, which can contribute to endothelial, vascular and neurovascular dysfunction (Kangralkar *et al.,* 2010)**.** Oxidative stress as a result of over production of reactive oxygen species and reduced antioxidant capacity is related to the onset and progression of type 2 diabetes mellitus. The consequences of oxidative stress are damage to DNA, lipids, proteins, disruption in cellular homeostasis and accumulation of damaged molecules.

1.4 Justification

In diabetic patients, an imbalance between reactive oxygen species production and antioxidant levels has been reported (Agarwal *et al*., 2003). There is lack of data on the actual status of antioxidants in type 2 diabetic patients. Information about the activities of enzymatic and non enzymatic antioxidants that are known to play useful roles in the

management of type 2 diabetes mellitus is required. The findings of the present study may support the therapeutic role of antioxidants in protecting islets from oxidative damage by reactive oxygen species in the period of the disease. Moreover, data on the oxidative stress markers have so far not been reported on Ghanaian subjects with type 2 diabetes.

1.5 Aim

The study was to evaluate lipid peroxidation and antioxidant status in type 2 diabetic patients.

1.6 Specific objectives

Specific objectives were as follows:

- To measure lipid peroxidation by the determination of plasma malondialdehyde (MDA), glutathione peroxidase (GSP–Px) levels and superoxide dismutase (SOD) levels in type 2 diabetic subjects.
- To estimate glycosylated hemoglobin (HbA_{IC}) levels and plasma glucose concentration in type 2 diabetic subjects.
- To investigate lipid profile in type 2 diabetic subjects.
- To investigate any association between glycaemic control and lipid peroxidation and antioxidant status.

CHAPTER TWO

2.0 Literature review

2.1.0 Definition of diabetes mellitus

Diabetes mellitus is a major global life-threatening disease with profound public health consequences. Although it has been centuries since diabetes was first recognized, it is still poorly understood and generally poorly managed. Diabetes mellitus describes a heterogeneous metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances in carbohydrate, fat and protein metabolism, resulting from defects in insulin secretion, insulin action, or both (WHO, 1999). It may be present with characteristics symptoms such as polydipsia, polyuria, polyphagia, weight loss.

2.1.1 Types of diabetes mellitus

There are two main types of diabetes mellitus (type 1 and type 2 diabetes mellitus), though there are other rare forms of it. Type 1 diabetes mellitus is characterized by insulin deficiency resulting from pancreatic beta cell destruction. The aetiology of type 1 diabetes mellitus is either immune mediated, related to either physical destruction of the pancreas (as in pancreatitis or pancreatic cancer) or idiopathic. However, the most prevalent form of diabetes, type 2 diabetes (which accounts for over 90% of all diabetes cases) presents as a spectrum of metabolic abnormalities with prominent insulin resistance and relative insulin deficiency (WHO, 1998). Other categories of diabetes, as suggested by Kuzuya and Matsuda (1997) are impaired glucose tolerance and gestational diabetes mellitus.

2.1.2 Diagnosis of Diabetes mellitus

The diagnostic criteria for diabetes mellitus have recently been revised by the Expert Committees of the American Diabetes Association (ADA, 2008) and the World Health Organization (WHO, 1999). The reviews have led to a lowering of the fasting glucose diagnostic criteria from ≥7.8 mmol/l to ≥7.0 mmol/l. The 2-hour criterion (≥11.0 mmol/L) however was unchanged. Additionally, a new category, impaired fasting glucose (IFG), was established and defined as fasting plasma glucose of 6.1 – 6.9 mmol/l. IFG is a metabolic state considered abnormal but not yet diagnostic of diabetes (ADA, 2008 and WHO, 1999).

2.1.3. Pathogenesis Type 2 diabetes mellitus

Under normal physiological conditions, plasma glucose concentrations are maintained within a narrow range, despite the wide fluctuations in supply and demand, through a tightly regulated and dynamic interaction between tissue sensitivity to insulin (especially in the liver) and insulin secretion in blood glucose concentration, which results in a rapid release of insulin by the β-cells within the islets of Langerhans. Insulin then binds to specific receptors in target peripheral tissues to produce its effects. Under normal conditions, insulin promotes the transport of glucose into the skeletal muscle and adipose tissue using Glut 4. In the liver, insulin acts by suppressing glycogenolysis and gluconeogenesis, and in adipose tissue, it inhibits lipolysis. Through the mechanism of decreasing hepatic and adipose glucose production, and by accelerating the uptake of glucose into peripheral tissues, the net effect of insulin's action is to lower blood glucose concentration (DeFronzo, 1997).

In persons with type 2 diabetes mellitus (type 2 DM) however; there is a gradual change in glucose homeostasis manifested as glucose intolerance and inefficient uptake of glucose from the blood by the peripheral tissues. The glucose intolerance is caused, in part, by an attenuated biological response to normal concentrations of insulin, a condition known as insulin resistance. In addition, type 2 DM is often associated with a progressive decrease in the sensitivity of the pancreatic ß-cells to glucose stimulation, with a subsequent decrease in insulin secretion. In time, there may be an increased demand for insulin due to worsening of insulin resistance. Eventually, the combined effects of increased insulin resistance and inadequate insulin secretion, in response to a glucose challenge will result in hyperglycaemia, which is a significant and prolonged increase in

blood glucose concentration (Bao *et al.*, 1996). Although the central figure is hyperglycaemia, the effect of diabetes mellitus is not limited to carbohydrate metabolism. Lipid and protein metabolism also play an important role in the progression of the disease. The abnormal glucose metabolism accounts for poorly regulated biochemical processes that glycosylate haemoglobin and other proteins and lipids throughout the body (Bao *et al*, 1996). The effects of the dysmetabolism in carbohydrates, lipids, and proteins include long-term damage, dysfunction and failure of various organs.

2.1.4. Aetiological factors underlying diabetes mellitus

Type 2 DM is a heterogeneous syndrome that results from an interaction between a genetic predisposition and environmental factors. In genetically predisposed individuals, the development and progression of type 2 diabetes mellitus appears to be facilitated by factors such as obesity, lack of physical activity, cigarette smoking, high intake of calorie-rich diets and low intake of fruits and vegetables (WHO, 1998, Halliwell, 2000, Hu *et al.*, 2001). Type 2 diabetes mellitus has a gradual and insidious onset, and some degree of hyperglycaemia may have been present for several years (10 – 20 years) before the diagnosis is confirmed (WHO, 1998). The pathogenesis of diabetes mellitus, regardless of its aetiology, progresses through several clinical stages during its natural history.

2.1.5 Complications of diabetes

Severe long term abnormalities can result such as eye complications, heart disease, kidney and foot problems if blood glucose levels are poorly controlled (Brophy *et al.*,

2007). These complications are of two types- microvascular complications that include retinopathy, nephropathy, neuropathy and peripheral vascular disorders and macrovascular complications that include cardiovascular and cerebrovascular disorders The complications of diabetes can involve multiple systems throughout the body that are susceptible to the detrimental effects of oxidative stress and apoptotic cell injury (Maiese *et al*., 2010).

2.1.7 Overview of Type 2 diabetes mellitus and oxidative stress

A body of evidence exists concerning the involvement of oxidative stress in the aetiology of diabetes and its subsequent complications (Sies, 2000). Oxidative stress arises in cells and tissues through the increased production of reactive oxygen species and/or from decreases in the antioxidant defense system (Gumieniczek *et al,* 2002). Several mechanisms seem to be involved in the generation of oxidative stress in the presence of elevated glucose concentrations. They include glucose auto-oxidation, enhanced glucose flux through the polyol pathway, and non- enzymatic and progressive glycation of proteins with subsequent increased formation of glucose derived advanced glycosylation end products (AGEs) (Bonnefont-Rousselot, 2000).

Although the underlying mechanisms of the pathogenesis of type 2 diabetes mellitus still remain to be determined, oxidative stress has been shown to be responsible, at least in part, for the progression of type 2 diabetes mellitus (Sies, 2000). This is supported by increased oxidative damage to lipids and DNA and impaired antioxidant defense systems in these subjects (Ruhe and McDonald, 2000 and Guerrero-Romeo *et al,* 2005). However,

it still remains to be elucidated whether oxidative stress acts as a prime cause, just emerges as a concomitant event or is a consequence of the onset of diabetes. Diabetic subjects may have a defective antioxidant status as a result of either increased use or reduced intake of antioxidant to fight the excessive free radical production that is associated with diabetes mellitus (Ahmad *et al.,* 2003). The sequelae of type 2 diabetes mellitus (hyperglycaemia and hyperlipidaemia) may not only be caused by elevated peroxidation, but pre-existing high rates of lipid peroxidation may predispose to and accelerate the development of diabetes especially upon exposure to a pro-oxidant challenge (Laight *et al.,* 2000). Such elevated rates may reflect poor antioxidant intake (Salonen *et al.,* 1995, Ford, 2001), inherited differences in rates of peroxidation and metabolism of lipid peroxides (Fachini *et al,* 2000, Halliwell, 2000). It is evident that with the fast progressing westernization of our society that is associated with a high intake of calorie-dense foods coupled with a low physical activity pattern many subjects in high-risk groups may be predisposed to a defective antioxidant status and an increased oxidative stress that may lead to the development of type 2 diabetes mellitus and its complications.

2.1.8. Free Radicals

The term "free radical" can be defined as any atom or molecule that contains an unpaired electron in its outer orbit that can exist independently (Halliwell, 1994; Halliwell and Gutteridge, 1998). As a result, they can be highly reactive, although this varies from

radical to radical, reacting locally to accept or donate electrons to other molecules to achieve a more stable state. Ground state oxygen (O_2) has two unpaired electrons each located in a different anti-bonding orbital. An oxidizing agent, such as O_2 is effective at absorbing electrons from the molecule it oxidizes (Halliwell and Gutteridge, 1998). The collective terms reactive oxygen species (ROS) or active oxygen species have been applied for a variety of free radicals and non-radicals intermediates (Halliwell, 1994).

2.1.9. Reactive Oxygen Species (ROS): Historic Background

It is generally accepted that molecular oxygen (O_2) was absent until the appearance of photosynthetic organisms (blue-green algae) about 2.5 billion years ago (Semenza, 2007**)**. The photosynthetic organisms convert carbon dioxide (CO_2) and water to glucose and O_2. In this molecular process, solar energy was transduced into chemical energy of carbon bonds. The water was oxidized to hydrogen peroxide (H_2O_2) to generate the reducing power (hydrogen atoms) required for photosynthesis and O_2 was released into the atmosphere as a by-product and the gradual build-up of O_2 in the atmosphere then drove the evolution of aerobic organisms (eukaryotes containing mitochondria) about 1.5 billion years ago (Semenza, 2007). In the mitochondria, glucose is oxidized to CO_2 and water. The reducing equivalents generated from oxidation of glucose pass electrons through the mitochondrial electron transfer chain (ETC) and the build-up of a proton gradient to drive the phosphorylation of adenosine 5'-diphosphate (ADP) to adenosine 5'-triphosphate (ATP), a process termed "oxidative phosphorylation" (Semenza, 2007**)**. These electrons ultimately react with O_2 to form H_2O. In this molecular process, O_2 serves as a terminal oxidant. This process is highly energy-efficient and is superior to respiratory pathways

that rely on other terminal oxidants such as fermentation (Semenza, 2007). However, the utilization of O_2 as a terminal substrate in oxidative phsophorylation may have potential dangerous consequences. When electrons pass through the mitochondrial ETC, a fraction of them (0.1-0.5%) escape from the ETC and combines with O_2 prematurely, resulting in the generation of the partially reduced product; superoxide (Imlay, 2003). Superoxide and other partially reduced forms of O_2 are termed "reactive oxygen species" (ROS). Because of their reactivity, the accumulation of ROS beyond the immediate needs of the cell may affect cellular structure and functional integrity, by bringing about oxidative modification (degradation) of critical molecules, such as DNA, proteins, and lipids (Imlay, 2003, Evans, 2003).

Research in the field of the "potential toxicity of oxygen" was raised by the publication of Gerschman's free radical theory of oxygen toxicity in 1954, which stated that the toxicity of oxygen is due to partially reduced forms of oxygen (Gerschman *et al.*, 1954). The toxic effects of oxygen were further supported by Denham Harman (1956), who hypothesized that oxygen radicals may be formed as byproducts of enzymic reactions *in vivo*.

In 1956, Denham Harman described free radicals as a Pandora's box of evils that may account for gross cellular damage, mutagenesis, cancer, and, last but not least, the degenerative process of biological aging. The second era of the science of free radicals in living organisms then entered another phase when researchers (McCord and Fridovich, 1969) discovered the enzyme superoxide dismutase (SOD) and finally convinced most colleagues that free radicals are important in biology. Numerous researchers were then

inspired to investigate oxidative damage inflicted by radicals on DNA, proteins, lipids, and other components of the cell. The third era began with the first report describing advantageous biological effects of free radicals when researchers provided further evidence that the superoxide anion, through its derivative, the hydroxyl radical, stimulates the activation of guanylate cyclase leading to formation of the "second messenger" cyclic guanosine monophosphate cGMP (Mittal and Murard, 1977).

2.2.0. Mechanisms of production of ROS

The reactive oxygen species (ROS) can be generated by both exogenous and endogenous means.

2.2.1 Endogenous production of ROS

In vivo, reactive oxygen species are produced both enzymatically and non-enzymatically. Enzymatic sources include NADPH oxidases located on the cell membrane of polymorphonuclear cells, macrophages and endothelial cells (Babior, 2000; Vignais, 2002; Babior *et al.*, 2002) and cytochrome P_{450}-dependent oxygenases (Coon *et al.*, 1992). The proteolytic conversion of xanthine dehydrogenase to xanthine oxidase provides another enzymatic source of $O_2^{-}\cdot$ and H_2O_2 (and therefore constitute a source of $OH\cdot$) has been proposed to mediate deleterious processes *in vivo* (Yokoyama *et al*., 1990). The non-enzymatic production of reactive oxygen species occurs when a single electron is directly transferred to oxygen by reduced coenzymes or prosthetic groups (for example, flavins or iron sulfur clusters) or by xenobiotics previously reduced by certain enzumes (for example, the anticancer agent adriamycin or the herbicide paraquat). The

mitochondrial electron transport chain contains several redox centres that may leak electrons to oxygen, constituting the primary source of $O_2^{-}\cdot$

2.2.2 Exogenous production of ROS

Natural diets contain plant food with large amounts of natural phenolic, as well as chlorogenic and caffeic acid, that may generate oxidants by redox cycling (Babior, 2000). Oxides of nitrogen in cigarette smoke (about 1000 ppm) cause oxidation of macromolecules and deplete antioxidant levels (Kiyosawa *et al*, 1990). Iron and copper salts also promote the generation of oxidizing radicals from peroxides through the Haber-Weiss reaction (Halliwell and Gutterridge, 1990).

2.2.3.0 Types of Reactive Oxygen Species

The most common ROS include: the superoxide anion ($O_2^{-}\cdot$), the hydroxyl radical ($OH\cdot$), hydrogen peroxide (H_2O_2), singlet oxygen ($1O_2$), and Peroxyl radicals and Ozone (O_3)

2.2.3.1. Superoxide anion (O_2^{-})

The superoxide is an anionic radical formed by the reduction of molecular oxygen through the acceptance of a single electron. The hydroperoxyl radical, which is unstable at physiological pH could also dissociate to superoxide. *In vivo*, the O_2^{-} is mainly produced by the ETC in the mitochondria and microsomes through electron leakage; a phenomenon that increases with an increase in oxygen utilization. Activated phagocytes also provide metabolic pathways for the production of superoxide radicals in response to bacterial infection (Babior, 2000).

2.2.3.2 Hydroxyl radical (HO^{-})

The hydroxyl radical may be produced by various procedures, including exposure to radiation or by decomposition of peroxynitrite (Beckman *et al*, 2002). Because of its low half-life, the direct action of the hydroxyl radical is confined to regions immediately in the vicinity of its formation. However, being the most reactive member of the ROS family, it can bring about extensive damage to different types of molecules, including proteins, nucleic acids, and lipids (Halliwell and Gutteridge, 1990). In DNA, the HO^{-} can induce several effects including base and sugar modifications, cross-linking between bases, cross-linking between DNA and protein, strand breaks, and formation of several adducts**.** The action of the hydroxyl radicals on proteins leads to extensive protein–protein cross-linking. This may be further aggravated in metalloproteins and metalloenzymes by transition metals, via the formation of hydroxyl radicals from their precursors, thus resulting in site-specific destruction of the critical regions of the molecule. Extensive studies on the oxidation of HO^{-} radicals have been carried out on membrane lipids in which the polyunsaturated fatty acids (PUFAs) are particularly vulnerable to oxidation (Halliwell and Gutteridge, 1990; Halliwell, 2000).

2.2.3.3 Hydrogen peroxide

Hydrogen peroxide and the superoxide radicals may undergo further transformations in the presence of transition metals (particularly iron and copper) to give rise to the highly reactive hydroxyl radicals, by the Haber–Weiss or Fenton reactions. This special property, combined with the membrane permeability of hydrogen peroxide, gives

superoxide and hydrogen peroxide the ability to affect the integrity of distant molecules within the cell (Halliwell and Gutteridge, 1990, Halliwell, 2000).

2.2.3.4 Singlet oxygen

Singlet oxygen ($1O_2$), largely known to be involved in photochemical reactions, is very reactive, although it does not contain unpaired electrons and therefore is not a free radical. It is formed *in vivo* by enzymatic activation of oxygen through several biological processes (Halliwell and Gutteridge, 1990), for example, through lipooxygenase activity during prostaglandin biosynthesis. It can also be produced by physicochemical reactions, such as energy transfer due to type II photosensitization, thermal decomposition of endoperoxides and dioxetanes, reaction of ozone with human body fluids and interaction between hydrogen peroxide and peroxynitrite.

Singlet oxygen is a very reactive ROS and induces various genotoxic, carcinogenic, and mutagenic effects through its action on polyunsaturated fatty acids (PUFAs) and DNA (Halliwell and Gutteridge, 1990, Sies H, 1993).

2.2.3.5 Peroxyl radicals

Several peroxyl radicals are produced primarily during lipid peroxidation, which is initiated by abstraction of a hydrogen atom from unsaturated lipids. Although lipid peroxidation has been found to play a useful role in some biological processes,

peroxidation of membrane PUFAs may adversely affect many functionally important processes, such as membrane fluidity, permeability, electrical potential, and controlled transport of metabolites across the membrane (Halliwell and Gutteridge, 1990, Halliwell, 2000).

2.2.4.0 Targets of ROS

Although O_2 is strongly oxidative with respect to its fully reduced form, water, it is a rare stable di-radical because of the kinetic restriction imposed by its two spin-aligned unpaired electrons. Molecular oxygen (O_2) can only react with transition metals or organic radicals with unpaired electron and is a very weak oxidant that cannot efficiently oxidize amino acid or nucleic acid (Imlay, 2003). However, its partially reduced products including superoxide, H_2O_2, and hydroxyl radical ($HO\cdot$) are more reactive. The anionic charge of superoxide inhibits its electrophilic activity toward electron-rich molecules and therefore superoxide could only oxidize few biomolecules such as enzymes containing the [4Fe-4S] clusters (aconitase or dehydratase as examples) (Imlay, 2003). The locally positively charged iron atom attracts superoxide electostatically and is therefore particularly susceptible to superoxide damage. Hydrogen peroxide (H_2O_2) is also a weak oxidant due to the stable oxygen-oxygen bond that limits its reactivity (Imlay, 2003). Although H_2O_2 can oxidize the cysteine (-SH) or methionine ($-SCH_3$) residues of proteins, the reaction is however very slow unless the cysteine residues are rendered more negatively charged by adjacent positively charged residues to form thiolate anion (Bindoli *et al*, 2008). The thiolate form of cysteine residue which is the most nucleophilic amino acid can therefore react readily with H_2O_2. Several protein kinases, phosphatases, and

transcription factors with important physiological functions contain thiolate residues that can be oxidized reversibly by H_2O_2. In contrast, the hydroxyl radical reacts readily with most biomolecules including lipids, amino acids, and nucleic acids (Imlay, 2003). Theoretically, the damaging effects of H_2O_2 are mainly due to its conversion to hydroxyl radical by Fenton reaction in the presence of free metals such as copper or iron and the superoxide radical can also react with another poor oxidant ; nitric oxide (NO·) to generate a very strong oxidant, peroxynitrite ($ONOO^-$), that reacts with most bio-molecules (Imlay, 2003).

The superoxide and hydroxyl radicals have relatively short half-life of between 10^{-6} and10^{-9} sec with very low intracellular concentration between 10^{-15} and 10^{-10} M (Winterbourn, 2008). On the other hand, H_2O_2 is relatively stable (half life: 10^{-5} sec) with higher intracellular concentration of 10^{-5}M (Winterbourn, 2008). Hydrogen peroxide is non-polar and can therefore diffuse freely across membranes with a very long diffusion distance of 1.5 mm in the presence of 2 mM glutathione (Winterbourn, 2008). The relative long diffusion distance of H_2O_2 and its ability to reversibly oxidize specific protein residues make it a potent molecule for signal transduction (D'Autréaux and Toledano, 2007).

2.3.0 Antioxidants

Antioxidants are described as substances that are able, at relatively low concentrations, to compete with other oxidizable substrates and, thus, to significantly delay or inhibit the oxidation of these substrates (Halliwell and Gutteridge, 1990, Halliwell, 2000). This broard definition includes the enzymes superoxide dismutase (SOD), glutathione

peroxidase (GPx), and catalase, as well as nonenzymic compounds such as a-tocopherol (vitamin E), ß-carotene, ascorbate (vitamin C), and glutathione. They may act possibly by removing or lowering the local concentrations of one or more of the participants in oxidising, such as oxygen, ROS, or metal ions such as Fe^{3+}, Cu^{2+} which catalyze oxidation, or interfering with the chain reaction that spreads oxidation to neighbouring molecules. They may also act by enhancing the endogenous antioxidant defenses of the cell. Hence, antioxidants may intervene at any of the three major steps: initiation, propagation, or termination of the oxidative process **(**Halliwell and Gutteridge, 1990; Halliwell, 2000).

2.3.1.0 Classification of action of Antioxidants

Several critical structures in the cell are protected by the availability of several types of antioxidants and may be classified according to their chemical nature and mode of action. The classes include: naturally-occurring enzyme antioxidants**,** preventive antioxidants and scavenging or chain-breaking antioxidants.

2.3.1.1 Naturally-occurring enzyme antioxidants

These antioxidants act on specific ROS after they are formed and degrade them to less harmful products. Examples of these antioxidants include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). SODs convert the superoxide radical to hydrogen peroxide, which although not a free radical by itself, exhibits its reactivity

via its precursor, the hydroxyl radical (Halliwell and Gutteridge, 1990, Halliwell, 2000). Catalase, SODs, and GPx constitute the major intracellular enzymic antioxidants, while the extracellular antioxidants are mainly of the preventive and scavenging types (Halliwell and Gutteridge, 1990; Halliwell, 2000).

2.3.1.2 Preventive antioxidants

Preventive antioxidants act by binding to and sequestering oxidation-enhancers and transition metal ions, such as iron and copper, which contain unpaired electrons and strongly accelerate free radical formation (Frei *et al*, 1988; Halliwell and Gutteridge, 1990; Halliwell, 2000). The preventive antioxidants include transferrin and lactoferrin , ceruloplasmin (binds copper), haptoglobins , hemopexin, and albumin (Frei *et al*, 1988; Halliwell and Gutteridge, 1990; Halliwell, 2000).

2.3.1.3 Scavenging or chain-breaking antioxidants

These antioxidants act by presenting themselves for oxidation at an early stage in the free radical chain reaction, producing low-energy molecules that are unable to propagate the chain further. Lipid-soluble and water-soluble antioxidant scavengers act in cellular environments that are either hydrophobic or hydrophilic, respectively. The major lipid-soluble scavengers are vitamin E (alpha-tocopherol), beta-carotene, and coenzyme Q (CoQ) while ascorbic acid, various thiols, uric acid, and bilirubin exhibit their functions in the aqueous medium (Frei *et al*, 1988; Halliwell and Gutteridge, 1990; Halliwell, 2000).

2.4.0. Overview of Oxidative Stress

The formation of reactive oxygen-containing molecules is a normal consequence of a variety of biochemical reactions. Therefore, there is the need to maintain a critical balance between the generation of these ROS and antioxidant defense. “Oxidative stress” occurs when there is imbalance between pro-oxidants and antioxidants in favor of the former. Because of the reactivity of the ROS, their accumulation beyond the immediate needs of the cell may affect both normal metabolism and physiology of cellular structure and functional integrity by bringing about oxidative degradation of critical molecules, such as DNA, proteins, and lipids (Sies, 1984 and Ames *et al.,* 1993).

Cells or tissues are in a stable state if the rates of ROS generation and scavenging capacity are essentially constant and in balance. Redox signaling mechanism suggests that this balance can be disturbed, either by an increase in ROS concentrations or a decrease in the activity of one or more antioxidant systems. In higher organisms, the activation of endogenous ROS-generating systems may induce such an oxidative event.

Similar responses may be induced by oxidative stress conditions generated by several other environmental factors such as exposure to radiation, cigarette smoking (Sies, 1984 and Frei *et al.,* 1988). If the initial increase in the level of ROS is relatively small, the antioxidative response may be sufficient to compensate for the increase in ROS and to reset the original balance between ROS production and ROS-scavenging capacity. Under certain conditions, however, the rate of ROS production is increased more strongly and persistently, and the antioxidative response may not be sufficient to reset the system to the original redox homeostasis. In such cases, the system may still reach equilibrium,

though the resultant quasi-stable state may now be associated with higher ROS concentrations (Sies, 1984 and Frei *et al.*, 1988).

2.4.1.0 Sources of oxidative stress in diabetes Mellitus

1) Glucose auto-oxidation: generation of superoxide and hydrogen.
2) Protein glycosylation and Amadori auto-oxidation: Generation of superoxide accompanied by glycoxidation product formation.
3) Decreased antioxidant status: Linked to sorbitol pathway activation and reduced levels of antioxidant vitamins and reduced glutathione.

2.4.1.1 Auto-oxidative Glycosylation

The reactions which occur when protein is exposed to glucose are considerably more complex than the simple addition of glucose to protein amino groups. Glucose can enolise and thereby reduce molecular oxygen, yielding oxidizing intermediates (Baynes, 1991). Free radical and hydrogen peroxide slowly produced by glucose auto-oxidation are the cause of structural damage which results, when protein is exposed to produce in vitro. Auto-oxidative glycosylation is a reasonable mechanism for the production of free radicals during glycosylation reaction. α-ketoaldehyde products of glucose auto-oxidation contribute to the covalent attachment of monosaccharide to protein.

2.4.1.2. Auto-oxidation of glucose and Amadori product

Work on protein damage by glucose in vitro focused on the ability of glucose to react with amino groups on the protein, forming initially a Schiff base and subsequently a

rearranged and more stable adduct, altering the charge, confirmation and molecular recognition of proteins (Baynes, 1991). It has been suggested that free radicals and hydrogen peroxide are produced slowly by glucose autoxidation are a substantial cause of structural damaged to protein exposed in vitro (Baynes, 1991). Oxidative reactions are thus critical for the production of glucose-induced protein alterations (Abuja and Albertini, 2001). Transition metals is able to react with lipids / proteins, leading to the formation of at least three different products: carboxymethly-lysine (CML), carboxymethyl-hydroxylysine and pentosidine Carboxymethyl-lysine and carboxymethyl-hydroxylysine are formed by oxidative cleavage of Amadori adducts (Baynes, 1991). Pentosidine is a fluorescent cross-link molecule formed between lysine and arginine residues in protein. Presence of CML strongly indicates that the transition metal catalyses oxidation in vivo. CML accumulation may indicate either an accumulation of oxidizable substrate or an increased level of transition metal in a form capable of catalyzing oxidation (Baynes, 1991). A large body of evidence suggests that advanced glycation end products (AGEs) are important pathogenic mediators of almost all diabetes complications (Baynes, 1991; Wolff, 1993; Prakash, 2007). Advanced glycated end products are found in retinal vessels of diabetic patients and levels correlate with those in sera of as well as with severity of retinopathy diabetic patients. Advanced glycated end products accumulate in peripheral nerves degrading the nerve conduction velocities and neuronal blood flow. Prolonged infusion of AGEs to non-diabetic rats has led to the development of renal morphological changes and development of significant amount of proteinuria. Studies also have shown that AGEs alter the function of

endothelial cells, macrophages and smooth muscles cells, leading to the formation and progressions of atherosclerosis (Peppa *et al*., 2003; Barnett *et al*., 1986).

2.4.1.3 Antioxidant status

Normally, free radicals are rapidly eliminate by enzymatic antioxidants, such as reduced glutathione and vitamin E and C. Diabetic subjects, however, have lower concentrations of these protective compounds (Maritim *et al*., 2003). Diminished antioxidant reserve may be due to competition for NADPH, which is a co-factor required to cycle the oxidized free radical scavengers back to their effective reduced form. NADPH is produced from the hexose monophosphate shunt, and one source of competition for NADPH comes from the sorbitol pathway. This pathway, which converts glucose to sorbitol, has been implicated in the pathogenesis of many diabetic complications. During conversion of glucose to sorbitol, NADPH is consumed. Increased flux through the polyol pathway causes increased NADPH utilization and leaves the tissue less able to resist oxidative stress (Fernandez –Real, 2002).

Biochemical *in vitro* studies suggest that glucose auto-oxidation may be a source of oxidative stress (Fernandez-Real, 2002; Peppa *et al*., 2003). Many studies have indicated that the rheological properties of diabetic erythrocytes are abnormal (Peppa *et al*., 2003; Prakash, 2007 Baynes, 1991). Oxidative stress may be responsible for the osmotic fragility and altered deformity of red blood cells, due to membrane lipid peroxidation. High glucose concentration is known to cause growth retardation and malfunctions. Oxygen radical scavenging enzymes (Superoxide dismuates, Glutathione peroxidase and Catalase) protect the embryo against this disturbed growth and development when added

to hyperglycemic culture media, suggesting that hyperglycaemia acts through production of free radicals.

2.5.0 Lipid peroxidation

The free radical oxidation of polyunsaturated fatty acid (PUFA) in biological systems is known as lipid peroxidation (Gutteridge, 1995). PUFA contains two or more double bonds, and the presence of an increasing number of double bonds in fatty acids makes it more susceptible to oxidative damage by free radicals and peroxidation (Symons and Gutteridge, 1998). Both monounsaturated and saturated fatty acids are much less reactive and do not usually participate in lipid peroxidation (Abuja and Albertini, 2001). In the non-enzymatic lipid peroxidation process, the addition of oxygen yields a lipid peroxyl radical, which is considered a hallmark of peroxidising lipids. Lipid peroxidation consists of mainly three processes, namely initiation, propagation and termination (reactions 1-4).

Initiation: $X^{\cdot} + RH \longrightarrow R^{\cdot} + XH$ (1)

Propagation: $R^{\cdot} + O_2 \longrightarrow ROO^{\cdot}$ (2)

$ROO^{\cdot} + RH \longrightarrow ROOH + R^{\cdot}$ (3)

Termination: $ROO^{\cdot} + ROO^{\cdot} \longrightarrow [ROOOOR] \longrightarrow NRP$ (4)

Lipid peroxidation can be initiated by any primary free radicals ($^{\cdot}OH$, $O_2^{-\cdot}$) of sufficient reactivity to substitute an allylic hydrogen atom from a reactive methylene group of PUFA side-chains. In the initiation step, polyunsaturated lipids (RH) form alkyl radicals ($R^{\cdot}$) which react very rapidly with oxygen to form peroxyl radicals ($ROO^{\cdot}$). In the

propagation step, and in the presence of more lipids, more peroxyl radicals (ROOH) a primary oxidation products. Propagation reactions can repeat themselves many times. Thus, an initial event triggering lipid peroxidation can be amplified with the availability of oxygen and PUFA side chains (Rice-Evans, 1994). Consequently, the accumulation of hydroperoxides and their subsequent decomposition to alkoxyl and peroxyl radicals can accelerate the chain reaction with PUFA leading to oxidative damage in cells, membranes and lipoproteins (Rice-Evans, 1994). Under such conditions where lipid peroxidation is continuously initiated, a termination reaction limits the extent of lipid peroxidation, yielding non-radical products (NRP), and destroying two radicals at the same time (Abuja and Albertini, 2001). In contrast, the potential consequences of the peroxidation of membrane lipids include loss of polyunsaturated fatty acids, loss of decreased lipid fluidity, altered membrane permeability, effects on membrane-associated enzymes, altered iron transport, and release of material from subcellular compartments and the generation of cytotoxic metabolites of lipid hydroperoxide (Rice-Evans, 1994).

2.5.1 Lipid peroxidation in diabetes

In diabetes mellitus abnormal increased levels of lipid, lipoprotein and lipid peroxides in plasma may be due to abnormal lipid metabolism (Alberti *et al.*, 1997). The structural changes are clearly oxidative in nature and oxidation of lipids, lipoproteins in plasma and cellular membrane are associated with the development of vascular disease in diabetes mellitus. Increased lipid peroxides in plasma might result from enzymatic processes activated by generalized vascular inflammation, leading to increased levels of

prostaglandins and lipoxygenase products. Alternatively, lipid peroxides might be formed by non-enxyamtic reactions of unsaturated lipids with superoxide radical, hydrogen peroxide, and adventitious metal ions in the circulation or extravascular space or at the surface of endothelial and phagocytic cells (Alberti, 1997). Lipid peroxidative damage may not be limited to the lipid compartment because lipid peroxides may cause browning and cross-linking of collagen and contribute to the development of fluorescence in plasma proteins and collagen in diabetes (Alberti, 1997). Increased glycation of collagen and plasma proteins in diabetes may stimulate the oxidation of lipids, which may in turn stimulate auto-oxidation reactions of sugars, enhancing damage to both lipids and proteins in the circulation and in the vascular wall, continuing and reinforcing cycle of oxidative stress and damage (Alberti, 1997).

2.5.6. Mechanisms of antioxidant actions on lipid peroxidation

In order to prevent overload of free radicals and peroxides, biological systems possess sophisticated antioxidant defensive mechanisms, which operate both in the intra and extracellular fluids that can prevent initiation or intercept lipid peroxyl radicals involved in the propagation phase. In human plasma, there are abundant binding proteins present that prevent metal-induced catalysis. In addition, cell membranes and lipoproteins contain lipophilic antioxidants, which react with lipid peroxyl radicals that eventually terminate the chain reaction (Abuja and Albertini, 2001).

$$LOO^{\cdot} + TocOH \longrightarrow LOOH + TocO^{\cdot}$$

$TocO^{\cdot}$ + Ascorbate ⟶ TocOH + Ascorbate radical

$TocO^{\cdot}$ + LH ⟶ $L^{\cdot}$ + TocOH

The tocopherol (Toc) radical ($TocO^{\cdot}$), located in lipid membranes, can be reduced to TocOH by ascorbate located in the aqueous phase. It is probable that physiological homeostasis requires a balance of antioxidants located in both aqueous and lipid phase. There are also synergistic interactions between the tripeptide glutathione (GSH) and vitamin E, which may involve membrane-bound enzymes. It has been demonstrated that under specific experimental conditions *in vitro*, in the absence of water-soluble antioxidants, $TocO^{\cdot}$ abstract $H^{\cdot}$ from an adjacent fatty acid and therefore act as a prooxidant. Consequently, removal of lipd hydroperoxides is an essential mechanisim for preventing such re-formation of free radicals, (Rice-Evans, 1994; Abuja and Albertini, 2001).

2.6.0. Biochemical markers of free radical mediated oxidative stress

2.6.1. Superoxide dismutase (SOD)

Superoxide dismutase is widely distributed in oxygen-metabolizing cells and has been reported to protect such cells against the deleterious action of superoxide radical (Misra and Fridovich., 1972). Role of SOD as antioxidant: SOD catalyzes the breakdown of the superoxide free radical ($O_2^{\cdot -}$) according to the reaction. $2O_2^{\cdot -} + 2H^{+} \xrightarrow{SOD} O_2 + H_2O_2$. Superoxide is formed by one-electron reduction of oxygen and has been identified as a

product in a number of biological reactions. The hydrogen peroxides so formed is removed by catalase or glutathione peroxidase (Fridovich, 1995).

Types of SOD:

1) Copper/Zinc superoxide dismutase (Cu/ZnSOD, or SOD1): Cu/ZnSOD is found in the cytoplasm and organelles of virtually all mammalian cells. It has a molecular mass of approximately 32,000KDa and has two protein subunits, each containing a catalytically active copper and zinc atom.

2) Extracelluar superoxide dismutase (ECSOD or SOD3): ECSOD is a Cu/ZnSOD but unlike its dimeric counterpart, it is tetrameric and glycosylated. This enzyme exhibits affinity for heparin and other acidic glycosamino-glycans because of a C-terminal heparin-bonding domain that is rich in basic amino acid residues (Fridovich, 1995) ECSOD is synthesized by only few cell types including fibroblasts and endothelial cells. ECSOD might play a role in the regulation of vascular tone because endothelial derived relaxing factor (nitric acid or a closely related compound) is neutralized in the plasma by superoxide (Wolff, 1993).

2.6.2. Malondialdyde (MDA)

MDA is an organic compound with the formula $CH_2(CHO)_2$. MDA mainly exists in the enol form. In organic solvents, the cis isomer is favored, whereas in water the trans isomer predominates. MDA is an end product of lipid peroxidation. Reactive oxygen species degrade polysaturated fatty acids, forming MDA. This compound is a reactive

aldehyde and is one of the many reactive electrophilic species that cause toxic stress in cells and form covalent protein adducts which are referred to as advanced lipoxidation end products (ALE). MDA reacts with deoxyadenosine and deoxyguanosine in DNA, forming DNA adducts, primarily pyrido [1,2- a]purin-10(3H)-one (M_1G), which is mutagenic. The quanidine group of arginine residues condenses with MDA to give aminopyrimidines. The production of MDA is used as a biomarker to measure the level of oxidative stress.

2.6.3. Glycated Hemoglobin (HbA_{1C})

Haemoglobin molecule consists of protein globin and heme as a prosthetic group. Globin part is made of four polypeptide chains. The hemoglobin tetramer, referred to as hemoglobin A (HbA), is best described as a pair of identical αβ dimmers ($\alpha_1\beta_1$) that associated to form the tetramer (Fridovich, 1995). Human adult hemoglobin usually consists of HbA_1 (HbA_2) (2.5%), and HbF (0.5%). Chromatographic analysis of HbA identifies several minor haemoglobins, namely $HbA_{1a,}$ HbA_{1b}, $HbA_{1c,}$ which are collectively called fast hemoglobin, glycohemoglobins, glycated hemoglobins or glycosylated hemoglobins. HbA_{1c} is formed by the condensation of glucose with N-terminal valine residue of each β-chain of HbA to form an unstable Schiff base, aldemine or pre- HbA_{1c}. This Schiff base may either dissociate or undergo an amadori rearrangenment to form a stable ketoamine. HbA_{1C} is the major fraction, constituting approximately 80% of HbA_1. Glycation may also occur at sites other than the end of β-chain, such as lysine residue or in α- chain. These are referred to as HbA_0. Glycation is

the non-enzymatic addition of a sugar residue to amino groups of proteins. Formation of glycated hemoglobin is irreversible and its level in blood depends on both life span of red blood cells (average 120 days) and blood glucose concentration. The glycosylated hemoglobin assay is a powerful research tool that is unique as it gives a retrospective index of glucose control over time in patients with diabetes (Nathan *et al*., 1984). Glycated hemoglobin concentrations represents the integrated values of glucose over preceding between 6 to 8 weeks since the rate of formation of glycated hemoglobin is directly proportional to the concentration of glucose in blood. Other advantage of glycated hemoglobin values for assessing glucose control are because these are free of day to day glucose fluctuations and unaffected by exercise or recent food ingestion (Burtis *et al*, 2006). The interpretation of glycated hemoglobin measurements will be affected by coincidental condition which reduces the life span of red blood cells, especially hemolytic anemia (Burtis *et al*, 2006). It is currently considered the best index of metabolic control for diabetic patients in clinical setting. It is as well a measure of risk for the development of micro and macrovascular complications (Calisti and Tognetti, 2005). Subjects with Type 2 diabetes mellitus with HbA_{1c} levels $\geq$ 7.5% have a 2.5 to 5 fold risk of developing micro vascular complications.

2.6.4. Glutathione Peroxidase

In normal biochemical processes, superoxide is detoxified rapidly by the enzyme superoxide dismutase (SOD), leading to the formation of hydrogen peroxide, which is subsequently converted to water in the cytosolic compartment of the cells, through the

action of the selenonenzyme glutathione peroxidase (GPx). Glutathione peroxidases constitute a family of enzymes which are capable of reducing a variety of organic hydroperoxides and lipid hydroperoxides to the corresponding hydroxyl compounds, utilizing glutathione, and/or other reducing equivalents, thus protecting cells from free radical mediated oxidative damage. Two types of glutathione peroxidases have been identified in human blood, namely classical cellular glutathione peroxidase in red blood cells and extracellular glutathione peroxidase in plasma (Brigelius-Flohe, 1999). Several studies, including both human (Aaseth and Stoa-Birketvedt, 2000; Varvarovska *et al*, 2003) and experimental animal (Mukherjee *et al*, 1998) have reported significant depletion of glutathione peroxidase in diabetes associated with enhanced lipid peroxidation.

CHAPTER THREE

3.0 METHODOLOGY

3.1.0. Study design

The study aimed at evaluating lipid peroxidation and antioxidant status in type 2 diabetes in Ghana. The study group consisted of 200 subjects made up of 100 subjects diagnosed with type 2 diabetes according to WHO (WHO, 1985) criteria who undergo routine clinical review at the out patients clinic of National Diabetes Management and Research Centre (NDMRC) at Korle-Bu teaching hospital. The control group consisted of 100 healthy non - diabetic age and gender-matched to the subjects. Of the one hundred type 2 diabetic subjects, 48 were males and 52 were females. Forty nine (49) of the control subjects were males and 51 were females. The selection criteria for the subjects were based on a structured questionnaire. The questionnaire was intended to obtain information on the subject's age, smoking habits, alcohol consumption, and duration of disease (type 2 diabetes mellitus), medications, and any other diseases. The study was approved by the Ethical Review Board Committee of University Ghana Medical School.

Participation in the study was voluntary. Informed consent was obtained from each participant. The sampling procedures were in accordance with the ethical standards of Helsinki Declaration of 1975.

3.2.0. Inclusion criteria

1. All volunteers aged between 35–55 years
2. Subjects who have ever been diagnosed as type 2 diabetes and being managed at NDMRC at Korle-Bu.
3. Apparently healthy individuals were used as controls; age and gender matched to the subjects.

3.3.0. Exclusion criteria

1. Pregnant women
2. Smokers
3. Uses of regular antioxidant supplements (vitamin C and folic acid) for at least one month before the start of the study.

3.4.0 Minimum sample size determination

The minimum sample size for the study was obtained using the formula:

$N = \frac{z^2 (p) (1-p)}{}$ $N = \frac{(1.96)^2 (0.036)(1-0.036)}{}$ $N = 63$

e^2 $(0.05)^2$

Where, z is the standard score for confidence level 1.96 for α = 5%,

p is the sample proportion which is 3.6% (Shaw *et al.*, 2010),

e is the minimum allowable error (5%).

For the purpose of this study, 100 subjects and age and gender-matched control subjects were recruited.

MATREIALS

Bathroom scale

Wall-mounted ruler

Sphygmomanometer and stethoscope

Human plate reader (Randox, UK)

Automated clinical chemistry analyzer (Randox, UK)

Blood glucose kit (Medsource Ozone Biomedicals Pvt, Ltd, India)

Total cholesterol kit (Medsource Ozone Biomedicals Pvt, Ltd, India)

Triglycerides kit (Medsource Ozone Biomedicals Pvt, Ltd, India)

High density lipoprotein cholesterol kit (Medsource Ozone Biomedicals Pvt, Ltd, India)

Glycosylated hemoglobin kit (Medsource Ozone Biomedicals Pvt, Ltd, India)

Superoxide dismutase assay kit (Bioassay systems, USA)

Glutathione peroxidase assay kit (Bioassay systems, USA)

Thiobarbituric reactive acid substance kit (Bioassay systems, USA)

3.5.0 Anthropometric variables

The subjects were weighed on a bathroom scale while barefooted and in light clothing and their height were measured with a wall-mounted ruler. BMI was calculated by dividing weight (kg) by height squared (m^2). Blood pressure was measured by qualified nurses using a mercury sphygmomanometer and stethoscope. Measurements were taken from the left upper arm after at least 5 minutes rest in a sitting position. Duplicate measurements were taken with a 5 min rest interval between measurements and the mean value was recorded in mmHg.

3.6.0. Sample preparation

Blood samples were collected from the ante cubital vein after 10-12 hours of overnight fast. Rubber tourniquet was applied for less than one minute and the site to be punctured cleaned with methylated spirit. Blood (8ml) was taken into separate vacutainer tubes. Two (2) milliliters of blood was dispensed into tubes containing fluoride oxalate, 2ml of blood was dispensed into ethylene diamine tetra acetic acid (EDTA) tubes whiles the rest

was dispensed into plain tubes and allowed to clot. The tubes were then placed in a centrifuge and spun at 3000 x g for 10 minutes to obtain the plasma and sera. Plasma glucose was measured immediately and the sera and plasma for the measurement of other biochemical variables and oxidative stress indices were stored at -80°C until analysis.

3.7.0 Metabolic biochemical markers for investigation

3.7.1. Estimation of blood glucose

Method: Trinder's method (Trinder, 1969)

Principle: Glucose + O_2 + H_2O $\xrightarrow{\text{Glucose oxidase}}$ Gluconate + H_2O_2

H_2O_2 + 4 – Aminoantipyrene + phenol $\xrightarrow{\text{POD}}$ Red quinoneimine complex + H_2O

Reagents	Concentration
Glucose oxidase	2000 U/L
Peroxidase	3250 U/L
4-Aminoantipyrene	0.52mmol/L
4-hydroxybenzoic acid	10 mmol/L
Phosphate buffer	110 mmol/L
Glucose standard	5.55mol/L

Assay procedure:

Thousand microlitres (1000 µL) of the glucose reagent was pipetted into a plain tube, and 10 µL of the plasma was addede. The content was well mixed and incubated for fifteen

minutes at room temperature. Finally, colour intensity was measured at 505nm using a spectrophotometer.

Samples were well mixed, incubated at 37^{o}C and the absorbances read at 505nm.

Calculation: Glucose (mmol/L) = $\frac{\text{Absorbance of test}}{\text{absorbance of standard}} \times$ concentration of standard (mmol/L)

3.7.2 Estimation of Glycosylated haemoglobin (HbA_{1C})

Method: Glycohemoglobin- Ion- exchange resin

Principle: A hemolyzed preparation of whole blood is mixed continuously for 5 minutes with a weak binding cation-exchange resin. During this time, the non-glycosylated hemoglobin binds to the resin leaving the supernatant containing the glycosylated hemoglobin. After the mixing period, a filter is used to separate the supernatant containing the glycosylated hemoglobin (GHb) from the resin. The GHb percentage is determined by measuring the absorbance at 415 nm of the GHb fraction and the total Hb fraction. The ratio of the absorbances gives the percentage of glycosylated hemoglobin (GHb).

Reagent composition**:**

Reagents	concentration

Cation-excahnge resin	8 mg/dl
Lysing agent	10Mm
Hemoglobin calibrator	10%

Assay procedure

Step 1 : hemolysate preparation

	Calibrator	Test
Lysing agent	500µl	500µl
calibrator	100µl	–
whole blood	–	100µl

Samples were well mixed and allowed to stand for 5 minutes till lysis was complete.

Step 2: separation of Glycohemoglobin

A 0.1ml of hemolysate was put into the marked ion-exchange resin tube and the separator inserted approximately 2 cm above the liquid level in the tube. The tube was rocked on the rocker for 5 minutes. The filter was pushed until the resin was firmly packed. The supernatant was put into appropriately labeled tube and the absorbance was read for Glycohemoglobin at 415 nm against deionised water blank.

Step 3: Total Hemoglobin fraction

	Calibrator	Test

Deionised water	5.0ml	5.0 ml
Calibrator hemolysate	20µl	–
Sample hemolysate	–	20µl

The sample was well mixed and the absorbance was read for total hemoglobin at 415 nm.

Calculation: % Glycohaemoglobin of sample = $\frac{\text{Absorbance of Glycohemoglobin}}{\text{Absorbance of Total Hemoglobin}} \times 5.2$

3.7.3. Total cholesterol

The method of analysis was first described by Trinder (1969). Cholesterol esters are broken down to cholesterol and fatty acids. The cholesterol is then oxidized to chole 4-en-3-one and hydrogen peroxide. The hydrogen peroxide is then hydrolyzed by a peroxidase to a red dye (quinoneimine).

Cholesterol Esters $\xrightarrow{\text{Cholesterol Esterase}}$ Cholesterol + Fatty acids

Cholesterol + O_2 $\xrightarrow{\text{Cholesterol Oxidase}}$ Cholest-4-en-3-one + H_2O_2

H_2O_2 + Hydroxybenzoic acid + 4-AAP $\xrightarrow{\text{Peroxidase}}$ Quinoneimine + H_2O

The intensity of the red colour produced is directly proportional to the concentration of cholesterol in the sample when read at 550 nm.

3.7.4. HDL-cholesterol

The method is based on phosphotungstate, a precipitating method. LDL, VLDL and chylomicrons are precipitated using phosphotungstic acid and leaving the HDL to react with cholesterol oxidase and cholesterol esterase. The enzymes selectively react with HDL to produce hydrogen peroxide through a Trinder reaction in the presence of Magnesium Chloride. The intensity of the red colour produced is directly proportional to the HDL-cholesterol in the sample when read at 505 nm.

$$\text{HDL} \xrightarrow{\text{Cholesterol esterase}} \text{Fatty acid} + H_2O_2$$

$$H_2O_2 + \text{4-AA} + MgCl_2 \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + 5\ H_2O$$

Assay procedure:

Two hundred microlitres (200µL) of the serum was dispensed into 200µL of the precipitating reagent. The mixture was well mixed and allowed to stand at room temperature for five minutes. It was then spun at 3000g for ten minutes. Thousand microlitres (1000µL) of the cholesterol reagent was pipetted into a plain tube. Hundred microlitres of the supernatant was dispensed into the working reagent, well mixed and incubated for ten minutes at room temperature. The optical density of the dye formed was proportional to the HDL-C concentration present in the sample and was measured at a wavelength of 505nm using a spectrometer.

3.7.5. Triglycerides

The method for the analysis is a modification of that of Trinder (1969). Triglycerides in the sample are reacted on by lipoprotein lipase (LPL) to glycerol and fatty acids. The glycerol is then phosphorylated by adenosine-5-triphosphate (ATP) to glycerol-3-phosphate (G -3-P) and adenosine-5diphosphate in a reaction catalyzed by glycerol kinase (GK). Glycerol-3-phosphate is then converted to dihydroxyacetone phosphate (DAP) and hydrogen peroxide by glycerophosphate oxidase (GPO). The hydrogen peroxide then reacts with 4-aminoantipyrine (4-AAP) and 2, 4, 6- Tribromo-3-hydroxy-benzoic acid (TBHA) in a reaction catalyzed by peroxidase (POD) to yield a red coloured quinoneimine dye. The intensity of the colour produced is measured at 505 nm and is directly proportional to the concentration of triglycerides in the sample.

Triglycerides + H_2O $\xrightarrow{LPL}$ Glycerol + Fatty acids

Glycerol + ATP $\xrightarrow{GK}$ G-3- P + ADP

G-3-P + O_2 $\xrightarrow{GPO}$ H_2O_2 + DAP

H_2O_2 + 4-AAP + TBHA $\xrightarrow{POD}$ Red Quinoneimine complex + H_2O + HCl

Assay procedure:

Thousand microlitres of the triglycerides reagent was dispensed into a plain tube and ten microlitres of the sera were pipetted into the plain tubes and were well mixed. The text was incubated for 15 minutes at room temperature. The intensity of the colour produced was measured at 505nm and was directly proportional to the concentration of Triglycerides in the sample.

3.7.6.Low density lipoprotein (LDL) cholesterol estimation

The Friedwald Equation

After estimating total cholesterol, triglycerides and HDL cholesterol, LDL cholesterol were calculated using Friedewald *et al.*, (1972) equation as given below. Results were expressed as mmol/l.

[LDL cholesterol] = [Total cholesterol] – [HDL cholesterol]- [Triglcerides]/2.2 (mmol/l)

3.8.0 Oxidative stress indices

3.8.1. Malondialdehyde

Method: By Satoh Method (Satoh, 1978)

Principle: Auto-oxidation of unsaturated fatty acids involve the formation of semi stable peroxides, which then undergo a series of reactions to form short chain aldehydes like MDA. One molecule of MDA reacts with two molecules of thiobarbituric acid with the elimination of two molecules of water to yield pink crystalline pigment with absorbance maximum at 535 nm.

Reagents:

1) 10% Trichloroacetic acid (TCA) : 25ml
2) TBA reagent: 25ml
3) Standard : 50 µL 15 Mm MDA

Standards

30 µM MDA	+ H_20	Volume	MDA (µM)
300 µL	+ 0µL	300	30.0
180 µL	+ 120 µL	300	18.0
90 µL	+ 210 µL	300	9.0
0 µL	+ 300 µL	300	0.0

To 0.1ml of serum, 0.2ml of 10% TCA was added and the tube was incubated on ice for 5 minutes. After centrifugation at 14000 x g for 5 minutes, 0.2 ml of the supernatant was transferred into a new labeled tube and diluted with a factor of 3. A 0.2 ml of TBA Reagent was added to the sample and standards. Tubes were vortexed and incubated at 100^{o}C for 60 minutes. Tubes were allowed to cool, vortexed and centrifuged at 14000 x g for 2 minutes. 0.1 ml was loaded onto well plate and the optical density was read at 535 nm.

Blank absorbance was subtracted from all standard and sample values. A change in absorbances at 535nm was plotted against standard concentrations and slope was determined from the standard curve.

The MDA concentration

Calculation: $[MDA] = \frac{\text{sample absorbance} - \text{blank absorbance}}{\text{Slope } (\mu M^{-1})} \times n \ (\mu M \text{ MDA equivalents})$

3.8.2. Glutathione peroxidase (GPX)

Glutathione peroxidase was measured by the method of Paglia and Valentine, (1967). In brief, glutathione peroxidase catalyses the oxidation of glutathione (GSH) by cumene

hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance at 340 nm was measured using a Human reader fully automated clinical chemistry analyzer. The reaction principle is given below.

$2GSH + ROOH \xrightarrow{GSHPx} ROH + GSSG + H_2O$

$GSSG + NADPH + H^+ \xrightarrow{GR} NADP^+$

Reagents:

Assay Buffer: 25ml GR Enzyme: 1 ml Glutathione: 240 µL NADPH: 40 µL

H_2O_2 Solution :100 µL calibrator : 100 µL Positive Control : 9 µL Glutathione Peroxidase (GPX).

Working reagent: 85 µL Assay buffer, 2 µL Glutathione, 2 µL 35 Mm NADPH and 8 µL GR enzyme were mixed to prepare the working reagent.

Standard preparation

A volume 0f 12 µL of the calibrator was mixed with 188 µL distilled water. And this mixture was used to prepare the standards.

Preparation of standards

No.	6mM Calibrator + H_2O	Volume (µL)	Equivalent NADPH
1	100 µL + 0 µL	100	6.0
2	60 µL + 40 µL	100	3.6
3	30 µL + 70 µL	100	1.8
4	0 µL + 100 µL	100	0.0

A 10 µL sample and 10 µL reconstituted GPX Positive Control pipetted separately into 96-well plate. In addition, a background control that only contains 10 µL Assay Buffer was included. A volume of 90 µL Working Reagent was quickly added to the Sample / control wells and mixed. An 8 µL H_2O_2 was diluted to a volume of 1000 and 100 µL of it was added to the Sample and Control wells and well mixed. Plate was read at 430 nm at time zero as OD_0 and read again at 4 min as OD_4. Blank value was subtracted from the standard values to give the change in absorbance (△OD). The △OD was plotted against standard concentrations and slope (S) was determined from the standard curve.

$\triangle OD_S$ (change in absorbance of sample) = OD_4 – OD_0

$\triangle OD_c$ (change in absorbance of control) = OD_4 – OD_0

GPX activity of Sample was calculated as:

$$\text{GPX Activity (U/L)} = \frac{[\triangle OD_S - \triangle OD_C] \text{ X } 100 \text{ x } n}{[\text{SLOPE X } 4\text{mins}]}$$

Where n is the sample dilution factor.

3.7.3 Superoxide dismutase

Superoxide dismutase was measured by the Method of Kuthan H, *et al.*, (1986).

Principle: superoxide is provided by the xanthine oxidase with a WST-1 dye to form a colored product. Superoxide dismutase scavenges the superoxide thus less superoxide is available for the chromogenic reaction. The color intensity at 440 nm is used to determine the superoxide activity in the sample.

Reagents

Assay Buffer: 20mL Diluent: 20Ml

SOD Enzyme: 120µL XO Enzyme: 120 µL

Xanthine: 600µL WST-1 600µL

Assay procedure

A 20µL SOD standard and samples were transferred into separate wells. A volume of 160µL of the working reagent was added to the wells. The XO enzyme was diluted with a dilution factor of 20. A 20 µL of a diluted XO enzyme was added to the well. Immediately, the wells were read at 440nm at time zero as OD_0. The plate well was incubated for 60 minutes at 25^0C in the dark and read at 440nm as OD_{60}.

Calculation: For each standard and sample well, calculations were done as;

Change in absorbance of sample (△ODs) = absorbance at 60 mins – absorbance at 0 min.

Change in absorbance of sample ($\triangle OD_{STDS}$) = absorbance at 60 mins – absorbance at 0 min

$\triangle\triangle OD = \triangle OD_{STD8} - \triangle OD_S$.

A graph of △△OD was plotted against [SOD](U/ML) and a standard curve was obtained.

The △△OD for sample was used to determine SOD activity of sample from the standard curve.

3.9.0. Statistical Analysis

All data were presented as mean ± standard deviation (SD) for 53 subjects in each group. The statistical package for social sciences (SPSS) version 16 was used for statistical evaluation. Significance of differences was determined using student t–test. Pearson's

correlation coefficient was determined within groups. Statistical significance was set at p-values < 0.05.

3.10.0 Expected outcome

The study will provide data on the oxidative stress biomarkers and also the findings of the present study may support a therapeutic role of antioxidants in protecting subjects with diabetes from oxidative damage by reactive oxygen species in the period of the disease.

3.11.0 Dissemination of the study outcome

The findings of the study will be communicated to the College of Health Sciences through seminars, scientific community and the general public through oral presentation and publication presentation in scientific journals. Also, the findings will be communicated to the Korle–Bu Teaching Hospital and the Diabetic Management and Research Centre for their consideration.

CHAPTER FOUR

4.0 Results

4.10. Anthropometric parameters in the subject populations.

The summary of the anthropometric characteristics of the subject populations are shown in table 4.10. There was no significance difference in the mean age of the type 2 diabetic and control subjects. The mean of the systolic blood pressure was significantly higher compared to the control subjects ($p<0.001$). Also the mean diastolic blood pressure in the type 2 diabetics were significantly higher compared with the healthy controls ($p<0.001$). Hypertension was prevalent among the type 2 diabetic subjects (60%). None of the healthy controls was hypertensive. The type 2 diabetic subjects have higher BMI than the healthy controls ($p <0.001$).

Table 4.10: The anthropometric characteristics of the study population

Parameter	T2DM(n=100)	Controls (n=100)		
	Mean ± SD	Mean ± SD	95% CI	p-value
Age (years)	50.25 ± 5.57	48.35±6.25		0.13
Disease duration (years)	7.70 ± 2.11	–		
SBP (mmHg)	135 ± 19.08	102.25 ± 9.60	113.7 - 123.7	0.0001
DBP (mmHg)	82.25 ± 12.5	70.9 ± 9.18	73.9 - 79.4	0.0001
BMI (Kg/m^2)	29.7 ± 3.2	22.1 ±2.4	24.2 – 28.9	0.0001

Table 4.10 shows the differences in anthropometric characteristics between type 2 diabetic and control subjects. The results are expressed as mean ±SD. SBP: systolic blood pressure, DBP: diastolic blood pressure, BMI: body mass index. p≤ 0.05 is significant.

4.11. Anthropometric characteristics between male and female in type 2 diabetic subjects.

The differences in anthropometric parameters between the men and women in type 2 diabetic subjects are depicted in table 4.11. The mean age difference between the male and female was not significant. Also there was no significance difference in the mean of the systolic and diastolic blood pressures between the male and female in type 2 diabetic subjects. Type 2 diabetic female subjects have a higher systolic blood pressure than the male though not significant. Female subjects had significantly higher BMI than males ($p<0.04$).

Table 4.11: Anthropometric characteristics between males and females in type 2 diabetic subjects.

Variable	Men (48)	Women (52)	
	Mean ±SD	Mean ±SD	p - value

Age (years)	49.42±4.4	51±5.49	0.34
Disease duration (years)	7.37±2.06	8 ±2.17	0.35
SBP (mmHg)	131.84±18.77	138.38±19.28	0.28
DBP (mmHg)	81.32±11.3	83.57±13.79	0.92
BMI (Kg/m^2)	25.3 ±2.8	37.8±3.6	0.04

Table 4.11 shows the differences in anthropometric subjects between the men and women in type 2 diabetic subjects. The results are expressed as mean ±SD. SBP: systolic blood pressure, DBP: diastolic blood pressure, BMI: body mass index, $p \leq 0.05$ was considered significant.

4.12. Anthropometric characteristics between males and females control subjects.

The differences in anthropometric parameters between the males and females control subjects are depicted in table 4.12. There were no significant difference in the mean age, diastolic blood pressure and BMI between the males and females amongst control subjects. However, female subjects showed a significantly higher mean systolic blood pressure compared to the males ($p<0.04$).

Table 4.12 Differences in anthropometric characteristics between males and females in control subjects.

Variable	Male (49) Mean ±SD	Women (51) Mean ±SD	p-value
Age (years)	45.61± 5.10	43.32 ±6.98	0.25
SBP (mmHg)	98.39 ±6.79	105.41±10.51	0.019
DBP (mmHg)	71.06 ±9.16	70.77± 9.41	0.57
BMI (Kg/m^2)	20.1 ± 1.4	21.1 ±3.4	0.50

Table 4.12 shows the differences in anthropometric characteristics between men and women in control subjects. The results are expressed as mean ±SD. SBP: systolic blood pressure, DBP: diastolic blood pressure, BMI: body mass index. $p \leq 0.05$ was considered significant.

4.13. Biochemical Parameters of the study populations.

Tables 4.13 shows the results of biochemical parameters of the study populations. The mean serum total cholesterol level was significantly higher among the type 2 diabetic subjects than in the healthy control subjects ($p < 0.001$). The mean serum level of LDL cholesterol in the type 2 diabetic subjects was significantly higher compared to the mean of the control subjects. The mean serum HDL- C was significantly higher in the healthy control subjects than type 2 diabetic subjects ($p \leq 0.0001$). The serum triglycerides concentrations in type 2 diabetic subjects were significantly higher than the healthy control subjects. The fasting plasma glucose level of type 2 diabetic subjects was significantly very high ($p < 0.001$) compared with healthy control subjects. There was a significance difference between the means of the glycated hemoglobin of the two groups ($p < 0.001$).

Table 4.13. Biochemical characteristics of type 2 diabetic and control subjects

Parameter	T2DM(n=100)	Controls (n=100)		
	Mean ± SD	Mean ± SD	95% CI	p-value
FPG	8.21±2.56	4.81±0.53	5.9 - 7.06	0.002
Hb_{AIC} (%)	8.82± 1.56	6.42±1.00	7.2 - 8.01	0.001
TC (mmol/L)	6.76±1.11	4.22±0.71	5.13 - 5.8	0.001
TG (mmol/L)	1.29 ± 0.34	0.94±0.23	1.03 - 1.18	0.001
HDL-C (mmol/L)	1.74±0.46	1.46±0.69	1.9 - 2.19	0.001

LDL-C (mmol/L)	3.96 ± 1.47	1.46±0.69	2.3 - 3.09	0.001

Table 4.13 shows the results for the biochemical parameters of the study population. Values are given as mean± standard deviation. Glycated hemoglobin, TC: total cholesterol, TG: Triglycerides, HDL-C: High density lipoprotein cholesterol, LDL-C: low density lipoprotein.

4.14. Biochemical characteristics of males and females in the control subjects.

As shown in table 4.14, there were no significance difference of the means of the biochemical variables between the males and females in the control subjects.

Table 4.14: Biochemical characteristic between males and females in control subjects

Parameter	Females (n=51)	Males (n=41)		
	Mean ± SD	Mean ± SD	95% CI	p-value
FPG	4.8±0.55	4.76±0.54	4.63 – 4.97	0.62
Hb_{AIC} (%)	4.82± 1.56	5.04±1.7	4.4 – 4.92	0.81
TC (mmol/L)	4.08±0.70	4.3±0.69	3.99 - 4.44	0.187
TG (mmol/L)	0.92 ± 0.212	0.91±0.25	2.16 – 2.57	0.81
HDL-C (mmol/L)	2.24±0.46	2.53±0.73	1.9 - 2.19	0.169
LDL-C (mmol/L)	1.54 ±0.72	1.4±0.66	1.24 - 1.68	0.532

Table 4.14 shows the results for the biochemical parameters between males and females in control subjects. Values are given as mean± standard deviation. Glycated hemoglobin, TC: total cholesterol, TG: Triglycerides, HDL-C: High density lipoprotein cholesterol, LDL-C: low density lipoprotein.

4.15 Biochemical characteristics between males and females in type 2 diabetic subjects

As shown in table 4.15, there were no significance difference of the means of the biochemical variables between the males and females in the control subjects. However, the mean of the serum triglycerides level in the women was significantly higher compared to the men ($p < 0.02$).

Table 4.15: Biochemical characteristics between males and females in diabetic subjects.

Parameter	Male (n=48)	Females (n=100)		
	Mean ± SD	Mean ± SD	95% CI	p-value
FPG	8.81±2.8	7.46±1.19	7.39 – 9.02	0.07
Hb_{AIC} (%)	8.7± 1.4	8.9±1.8	8.3 – 9.3	0.711
TC (mmol/L)	6.9±0.9	6.6±1.2	6.4 - 7.1	0.43
TG (mmol/L)	1.36±0.37	1.24±0. 27	1.18 -1.39	0.833
HDL-C (mmol/L)	1.7±0.45	1.72±0.48	1.59 - 1.88	0.849
LDL-C (mmol/L)	4.01±1.14	3.92 ± 1.54	3.49 – 4.4	0.39

Table 4.15 shows the results for the biochemical parameters between males and females in diabetic subjects. Values are given as mean± standard deviation. Glycated hemoglobin, TC: total cholesterol, TG: Triglycerides, HDL-C: High density lipoprotein cholesterol, LDL-C: low density lipoprotein.

4.16. Oxidative stress indices of the study subjects.

Table 4.16 shows the distribution of oxidative stress indices in the type 2 diabetic and control subjects. The mean value of malondialdehyde was significantly increased in the type 2 diabetic subjects compared with control subjects ($p < 0.001$). There was a

significant reduction in the activity of SOD in type 2 diabetic subjects ($p < 0.001$). The mean serum glutathione peroxidase was significantly higher in the control subjects than the type 2 diabetic subjects ($p<0.001$).

Table 4.16 Oxidative stress indices of study subjects

Variable	Type 2 diabetic subjects (n=53)	Controls (n=53)		
	Mean ±SD	Mean ±SD	95% CI	p- value
MDA (µM)	4.40 ± 1.96	2.75±1.05	3.18 – 3.96	0.001
SOD (U/Ml)	3.80 ±1.64	10.39±2.55	6.2 – 7.97	0.001
GPx (U/L)	129.96±16.75	174.20±36.02	144.13 – 160.02	0.001

The table 4.16 shows the oxidative stress parameters of the study subjects. The results are expressed as mean ±SD. MDA: Malondialdehyde, SOD: Superoxide dismutase, GPX: Glutathione peroxidase.

4.17. Oxidative stress indices between males and females in control subjects.

The oxidative stress indices between women and men in control subjects are shown in table 4.17. The men control groups have a higher MDA levels than the women but not significant. There was no significant difference in means of the SOD in control subjects in terms of gender. The men has significantly more levels of glutathione peroxidase activity than the women in the control subjects ($p<0.04$).

Table 4.17 Oxidative stress indices between males and females in control subjects

Variable	Females (n=51)	Males (n=49)		
	Mean ±SD	Mean ±SD	95% CI	p- value

MDA (µM)	2.6± 0.9	2.75±1.05	2.41 – 3.05	0.45
SOD (U/Ml)	10.63±1.64	10.39±2.55	9.57 – 11.21	0.32
GPx (U/L)	168.48±29.57	181.19±42.1	162.68 – 185.02	0.04

The table 4.17 shows the differences of oxidative stress parameters between males and females in the healthy control subjects. The results are expressed as mean ±SD. MDA: Malondialdehyde, SOD: Superoxide dismutase, GPX: Glutathione peroxidase, $p \leq 0.05$ was considered significant.

4.18. Oxidative stress indices between males and females in type 2 diabetic subjects.

The oxidative stress indices between women and men in type 2 diabetic subjects are shown in table 4.19.The men have a higher MDA levels than the women but not significant. There was no significant difference in means of the SOD in control subjects in terms of gender. The men has significantly more levels of glutathione peroxidase activity than the women in the control subjects ($p<0.04$).

Table 4.18: Oxidative stress indices between men and women in diabetic subjects

Parameter	Females(n=51) Mean ± SD	Males (n=49) Mean ± SD	95% CI	p-value
MDA (µM)	4.16±1.49	4.65±2.39	3.77 – 5.02	0.52
SOD (U/Ml)	3.8±1.38	3.79±2.80	3.27 – 4.31	0.46
GPx (U/L)	123.49±11.52	133.78±20.7	124.6 – 135.3	0.09

The table 4.18 shows the differences of oxidative stress parameters between males and females in the of type diabetic subjects. The results are expressed as mean ±SD. MDA: Malondialdehyde, SOD: Superoxide dismutase, GPX: Glutathione peroxidase, $p \leq 0.05$ was considered significant.

4.19. Associations between anthropometric variables in type 2 diabetic subjects.

Tables 4.19 shows the Pearson's correlation table for the anthropometric variables in the type 2 diabetic subjects. There was a weak positive correlation between age and systolic blood pressure ($r = 0.265$, $p < 0.0001$) and a weak negative correlation between age and BMI. Age showed a positive correlation with diastolic blood pressure in diabetic population but not significant.

Table 4.19 Pearson's correlation table of anthropometric variables in the diabetic subjects

	AGE		SBP		DBP	
	r	p	r	p	r	p
AGE						
SBP	0.290	0.001				
DBP	0.006	0.34	0.583	0.001		
BMI	-0.145	0.42	0.293	0.04	0.357	0.03
DD	0.014	0.82	0.162	0.72	0.127	0.62

Table 4.19 shows the Pearson's correlation table of anthropometric variables in the diabetic subjects. P values ≤ 0.05 was considered significant: SBP: systolic blood pressure, DBP: diastolic blood pressure, BMI: body mass index, DD: duration of disease (years). r: regression coefficient, p: probability value.

4.20. Associations between anthropometric and biochemical variables in type 2 diabetic subjects.

Tables 4.20 shows the Pearson's correlation table of anthropometric and biochemical variables in diabetic subjects. There was no significant correlation between age and the other variables. BMI correlated positively with total cholesterol but correlated negatively with HDL cholesterol.

4.20. Associations between anthropometric and biochemical variables in type 2 diabetic subjects.

	FPG		HbA1c		TC		TG		HDL		LDL	
	r	p	r	p	r	p	r	p	r	p	r	p
AGE	0.02	0.78	0.03	0.8	0.015	0.71	0.165	0.49	0.035	0.81	0.066	0.38
SBP	0.210	0.65	0.227	0.143	-0.013	0.83	0.205	0.67	-0.165	0.57	0.015	0.43
DBP	0.181	0.23	-0.088	0.79	0.007	0.45	0.149	0.45	-0.074	0.14	0.005	0.88
BMI	0.074	0.56	0.096	0.29	0.41	0.001	0.082	0.22	-0.319	0.03	0.066	0.34
DD	0.247	0.12	0.096	0.68	-0.215	1.19	0.077	0.28	-0.165	0.23	0.004	0.48

Table 4.20 shows the Pearson's correlation table of anthropometric variables in the diabetic subjects. P values ≤ 0.05 was considered significant: SBP: systolic blood pressure, DBP: diastolic blood pressure, BMI: body mass index, DD: duration of disease (years). r: regression coefficient, p: probability value

4.20: Associations between anthropometric variables and oxidative stress indices in type 2 diabetic subjects.

Tables 4.20 shows the Pearson's correlation tables of anthropometric variables and oxidative stress indices. The anthropometric variables showed negative correlation with plasma antioxidants. The BMI and duration of disease correlated significantly with the SOD but was not statistically significant with GPx. BMI association with MDA was statistically significant ($r = 0.532$, $p < 0.0001$).

Table 4.21 Pearson's correlation table of anthropometric variables and oxidative stress indices in the diabetic subjects.

	MDA		SOD		GPx	
	r	p	r	p	r	p
AGE	0.014	0.47	- 0.034	0.45	- 0.245	0.26
SBP	-0.126	0.19	0.081	0.48	0.235	0.29
DBP	-0.1379	0.34	0.069	0.52	0.20	0.68
BMI	0.532	0.0001	-0.610	0.0001	-0.082	0.24
DD	0.291	0.18	- 0.379	0.03	-0.085	0.46

Table 4.20 shows the Pearson's correlation table of the association between the anthropometric variables and the oxidative stress indices in the type 2 diabetic subjects. P values ≤ 0.05, SBP: systolic blood pressure, DBP: diastolic blood pressure, BMI: body mass index, DD: duration of disease (years). SOD; superoxide dismutase, GPx; Glutathione peroxidase, MDA; malondialdehyde. r: regression coefficient, p: probability value.

4.22. Associations between biochemical variables in type 2 diabetic subjects.

Tables 4.22 shows the Pearson's correlation table of biochemical variables in type 2 diabetic population. Most of the biochemical variables correlated well with each other in type 2 diabetic population. There was a positive correlation between plasma glucose and total cholesterol.

Table 4.22: Pearson's correlation table of anthropometric variables and biochemical variables in the diabetic population

	FPG		HbA1c		TC		TG		HDL	
	r	p	r	p	r	p	r	p	r	p
FPG										

HbA1C	0.18	0.31								
TC	0.206	0.24	-0.085	0.61						
TG	0.491	0.001	-0.041	0.81	0.385	0.001				
HDL	0.02	0.56	-0.159	0.23	0.037	0.62	-0.076	0.48		
LDL	0.36	0.03	0.02	0.83	0.33	0.001	0.38	0.001	-0.284	0.01

Table 4.22 shows the Pearson's correlation table of anthropometric variables in the type diabetic subjects. $p \leq 0.05$, was considered significant. SBP: systolic blood pressure, DBP: diastolic blood pressure, BMI: body mass index, DD: duration of disease (years). r: regression coefficient, p: probability value.

4.23. Associations between biochemical variables and oxidative stress indices

Table 4.23 shows the Pearson's correlation table of biochemical variables and oxidative stress indices. There was a significant negative association between plasma glucose and the plasma antioxidants and a significant positive correlation between plasma glucose and malondialdehyde in type 2 diabetic population. A similar relationship was observed with total cholesterol, LDL-cholesterol and triglycerides. This suggests that higher levels of plasma glucose and lipid lead to a depletion in the antioxidant defenses and a consequential increase in lipid peroxidation.

Table 4.23 Pearson's correlation table of biochemical variables and oxidative stress indices in the diabetic subjects.

	MDA		SOD		GPx	
	r	**p**	**r**	**p**	**r**	**p**
FPG	0.614	0.001	-0.511	0.001	-0.341	0.001
HbA1C	0.05	0.45	0.2094	0.67	0.281	0.03
TC	0.395	0.001	-0.349	0.001	-0.084	0.37
TG	0.228	0.03	-0.327	0.002	-0.031	0.42

HDL	-0.312	0.0001	-0.070	0.24	0.296	0.02
LDL	0.295	0.04	-0.282	0.0001	-0.453	0.025

Table 4.23 shows the Pearson's correlation table of biochemical variables and oxidative stress indices in the diabetic population. $p \leq 0.05$ significant. SBP: systolic blood pressure, DBP: diastolic blood pressure, BMI: body mass index, DD: duration of disease (years), r: regression coefficient, p: probability value.

4.24. Associations between oxidative stress indices in type 2 diabetic subjects.

Tables 4.24 shows the Pearson's correlation table of oxidative stress indices in type 2 diabetic. The plasma antioxidants correlated positively with each other in type 2 diabetic subjects suggesting a synergistic relationship in their antioxidant mechanism. The plasma superoxide dismutase and glutathione peroxidase showed a significantly negative correlation with malondialdehyde in type 2 diabetic subjects.

Table 4.24: Pearson's correlation table between oxidative stress indices in the diabetic subjects

	MDA		SOD	
	r	p	r	p
MDA				
SOD	-0.603	0.001		
GPx	-0.564	0.001	0.423	0.002

Table 4.24 shows the Pearson's correlation table of oxidative stress parameters in the type 2 diabetic subjects. $P \leq 0.005$ was considered, SOD: superoxide dismutase, MDA: Malondialdehyde, GPx: Glutathione peroxidase.

CHAPTER FIVE

5.0 Discussion

Overweight and obesity are alarming problems in affluent countries and are increasingly becoming a health burden in developing countries. Both of them are risk factors in type 2 DM and hasten cardiovascular complications mainly due to altered lipid profile. This study aimed at evaluating lipid peroxidation and antioxidant status in type diabetes mellitus.

The results of this study showed that type 2 diabetic subjects had significantly higher mean blood systolic and diastolic pressures. In this study BMI was measured and examined in relationship with lipid profile and other parameters. The results in this study show that subjects with type 2 DM were obese and had elevated serum levels of lipids. Also, body mass index was significantly higher in type 2 diabetic subjects than control group. These results agree with Stefanovic *et al*., (2010), where elevated levels of BMI were recorded in type 2 diabetic subjects.

These findings may be as a result of significantly increased levels of total cholesterol, low density lipoprotein (LDL), cholesterol and triglycerides but significantly decreased level of high density lipoprotein (HDL) cholesterol in type 2 diabetic subjects compared with control subjects. This finding agrees with Soliman, (2008). It is known that increase in triglyceride stores is always associated with a linear increase in the production of cholesterol (Astrup and Finer, 2000, Liu and Manson, 2001). Obesity is an important risk

factor for insulin resistance in pre-diabetic patients and diabetic complications and in the development of oxidative stress (Grundy, 2006).

On gender, no significant difference was observed in SBP and DBP between male and female type 2 diabetic subjects, though females had higher SBP and DBP compared with males subjects. This may be due to the significantly higher BMI in females compared with males (Table 4.11).

The study also showed that type 2 diabetes had significantly higher plasma glucose levels compared with control subjects. Furthermore, increased glycated haemoglobin level was observed in type 2 diabetes mellitus subjects compared with control subjects (Table 4.13). This results in increased lipid peroxidation and oxidative stress in type 2 diabetes mellitus compared with control subjects.

Studies have shown that hyperglycaemia leads to overproduction of reactive oxidants through several pathways that may lead to destruction of various macromolecules in the body including lipids through the mechanism of oxidative stress (Evans *et al*., 2003). The positive association between plasma glucose and lipid peroxidation as shown in Table 4.23 supports this biological mechanism.

In this present study, glycated haemoglobin levels were significantly increased in type 2 diabetes mellitus compared with controls and showed positive correlations with total cholesterol, LDL-cholesterol, triglycerides and negative correlation with HDL-cholesterol in DM patients. This findings is in agreement with studies by Fikree *et al*., (2006) and Veerma *et al*., (2006), who found that glycated haemoglobin levels correlated

positively with total cholesterol, LDL-cholesterol, triglycerides but negatively correlated with HDL-cholesterol.

Poor glycaemic control leads to increase in the lipid hydroperoxide and decrease in the total thiols. Hyperglycaemia-induced overproduction of superoxide ion by mitochondrial electron transport chain is the single driving force of the major molecular mechanisms implicated in the glucose-mediated vascular damage. Glycated haemoglobin levels above 7% may trigger oxidative stress, which contribute to tissue damage through oxidation of low-density lipoprotein and exacerbation of endothelial dysfunction, leading to the development and progression of vaculopathies (Fowler *et al*., 2010).

The high concentration of serum lipids in the type 2 diabetic subjects is mainly as a results of increased mobilization of free fatty acids from peripheral depots, due to loss of the inhibitory action of hormone sensitive lipase (Pasupathi *et al*., 2009). The low level of HDL-cholesterol, which exerts anti-atherogenic and antioxidative effects when present in sufficient amounts, is a key feature of type 2 diabetes.

Peroxidation of polyunsaturated fatty acids in blood produces malondialdehyde (MDA) that leads to oxidative damage. Malondialdehyde is frequently used to determine the oxidant/antioxidant balance in diabetic patients (Altomare *et al*., 1992; Gallou *et al*., 1993), MDA is a good indicator for evaluating oxidative stress in diseases like diabetes mellitus.

In this study, serum MDA levels in type 2 diabetic subjects were significantly elevated compared with control subjects. Furthermore, there was a positive correlation between total cholesterol, triglycerides, LDL-cholesterol and MDA. This finding agrees with studies by Lamarch *et al*., (1996) and Grundy, (2006), which reported significant elevated levels of MDA in type 2 diabetic subjects. In their studies, they also observed that, MDA correlated positively with total cholesterol, triglycerides and LDL- cholesterol. However, Mahbood *et al*., (2005) and Poon *et al.*, (2004) did not find any correlation between MDA and lipid levels. The finding in this study suggests that higher levels of plasma glucose and lipid levels. The finding in this study suggests that higher levels of plasma glucose and lipid lead to an increase in oxidant levels and a consequential decrease in antioxidant levels and therefore, increased lipid peroxidation. The increased level of MDA could also be as a result of increased glycation of proteins in diabetes mellitus. The glycated protein may themselves act as a source of free radicals.

The exact mechanism by which the elevated blood glucose leads to membrane lipid per oxidation is not known. Some studies have shown that glucose can enolise and then reduce molecular oxygen to give α- keto aldehydes, hydrogen peroxide and ROS. Hydrogen peroxide formed by superoxide dismutation regenerates the catalytic metal oxidation state and produces hydroxyl radicals. The ROS formed causes peroxidative breakdown of phospholipid fatty acids and accumulation of MDA (Jane *et al*., 1989). Elevated levels of MDA could also be due to alteration in the function of erythrocyte membranes.

Numerous reports indicate variations in the levels of antioxidants in diabetic subjects (Lee *et al*., 1995; Young *et al*., 1991; Obrosova *et al*., 2002). The reports about the SOD activity in diabetes are controversial, with some authors reporting no change in SOD activity (Dincer *et al*., 2002; Van *et al*., 2003) while others reported increased (Kesavulu *et al*., 2000; King *et al*., 2004) and others decreased SOD activity (Dandona *et al.*,1996 ; Bhatia *et al*., 2003). In this present study, a significant decrease in SOD activity in type 2 diabetic subjects when compared with control subjects were observed. SOD is an endogenously produced intracellular enzyme that plays a key role in defending the cell against oxygen free radical. The observed significant decrease in SOD activity in type 2 diabetic subjects could be attributed to increase production of superoxide free radical, elevation of other reactive oxygen species such as hydroxyl radical and H_2O_2. Intensification of lipid peroxidation processes also contribute to the declined SOD levels.

Hyperglycaemia contributes to oxidative stress by virtue of the fact that monosaccharides and glycolytic intermediates can generate oxidative reactants. Glucose can enolize and thereby reduce molecular oxygen under physiological conditions in the presence of traces of transition metals yielding oxidizing agents like H_2O_2. Partially reduced oxygen intermediates like superoxide anion radical and hydrogen peroxide generated in the course of this autoxidation associated with glycation contribute to the oxidative stress. This is suggestive of the fact that increased auto-oxidative glycation of hemoglobin leads to enhanced generation of free radicals like the superoxide anion, thereby causing the depletion of SOD. It is suffice to suggest that hyperglycaemia influences the etiopathogenesis of diabetes in more than one way (Tare *et al.,* 1999).

There is no consensus on the activity of GPx in the serum of diabetic subjects. Aydin *et al*., (2001) reported no change, while Kelly, (2000) reported increase in activity of GPx. In this study, a significantly deceased GPx activity was observed in type 2 diabetic subjects compared with the controls. The observed decrease in glutathione peroxidase level in type 2 diabetic subjects is indicative of decreased scavenging capacity of glutathione-dependent antioxidant defensive system against hyperglycaemia and lipid peroxidation processes in type 2 diabetic subjects. Glutathione peroxidase is one of the enzymes responsible for the removal of H_2O_2 produced as a part of cellular metabolism. Increased MDA levels and increased lipid peroxidation, together may act to inhibit GPx activity (Tur *et al*., 2002; Mahboob *et al*., 2005; Singhania *et al*., 2008).

In this study, the plasma antioxidants correlated positively with each other in type 2 diabetic subjects suggesting a synergistic relationship in their antioxidant mechanism.

This study has examined the relationship between gender and oxidative stress. Mean value of SOD and GPx was higher in both healthy control females, and in females with diabetes. These antioxidants levels were slightly decreased in diabetic males compared with diabetic female subjects. However, there was no significant difference. This clearly shows that diabetic subjects, irrespective of the sex, were exposed to an increased oxidative stress.

Conclusion

The results of the present study suggest that there is raised malondialdehyde levels, decreased activities of superoxide dismutase and Glutathione peroxidase along with increased body mass index and deranged lipid profile in type 2 diabetes mellitus subjects.

Limitation of the study

The study being cross-sectional, did not report causality effect- the study did not follow the patients to monitor response with their medications. This is not a nation-wide study on diabetic subjects although the setting is NDMRC at Korle-Bu. Also the exogenous antioxidants were not determined.

Recommendation

Additional work is needed to confirm whether an association exist between antioxidant depletion and complications of type 2 diabetes mellitus and that more oxidative stress markers be evaluated to ascertain the role of oxidative stress in type 2 diabetes mellitus.

REFERENCE

Aaseth J, Stoa-Birketvedit G. (2000). Glutathione in overweight patients with poorly controlled type 2 diabetes. *JTrace Elem Exp Med* **13**: 105-111.

Abuja K and Albertini, C. (2001). Leukocyte lipid peroxidation, superoxide dismutase, glutathione peroxidase, and serum and leukocyte vitamin C levels of patients with type II diabetes mellitus. *Clin Chim Acta,* **244**: 221-227.

Agarwal A. Saleh RA, Bedaiwy MA, (2003). Role of reactive oxygen species in the pathophysiology of human reproduction, fertility and sterility; **79**: 829-843.

Ahmad M, Ahmad K.M, Khan A.S. (2003). Naturally occurring antioxidant vitamin levels in patients with type 2 diabetes. *JAyub med Coll Abbotabad*; **15**(1).

Altomare E, Vendemiale G, Chicco D (1992). Increased lipid peroxidation in type 2 poorly controlled diabetic patients. *Diabetes Metabolism* **18:** 264-271

American Diabetes Association (ADA) (2010). Test of glycaemia in diabetes. *Diabetes Care* **25**: S29-S99.

American Diabetes Association (ADA). (2008). Diabetic nephropathy. *Diabetes Care* **20** (Supp 1): S24-S25

Ames, B.N, Shigenaga, M.K., Hagen T.M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci*. USA; **90**, 7915- 7922.

Amoah AG, Owusu SK, Adjei S. (2002). Diabetes in Ghana: a community based study in Greater Accra. *Diabetes Res Clin Pract*; **56**(3): 197-205.

Amoah AG. (2003). Sociodemographic Variations in Obesity among Ghanaian Adults. *Public Health Nutrition*; **6**(8): 751-757.

Aspray T.J, Mugushi F, Rashid S, Whiting D, Edwards R, Alberti K.G, Unwin N.C. (2000) Rural and urban differences in diabetes prevalence in Tanzania, the role of obesity, physical inactivity and urban living, Trans. R. Soc. *Trop. Med. Hyg*; **94**:637–644.

Astrup A and Finer N (2000): Redefining type 2 diabetes: 'diabesity' or 'obesity dependent diabetes mellitus'? *Obes Rev*; **1:**57-59.

Aydin A, Orhan H, Sayal A, Ozata M, Sahin G, Isimer A. (2001). Oxidative stress and nitric oxide related parameters in type II diabetes mellitus: effects of glycemic control. *Clinical Biochem;* **34:65**-70.

Aybek H, Aybek Z, Rota S, Sen N, Akbulut M. (2004). The effects of diabetes mellitus, age, and vitamin E on testicular oxidative stress. *Fertil Steril*; **90**: 755-60.

Babior BM. (2000). Phagocytes and oxidative stress. *Am J Med*; **109**:33-34.

Bao W, Srinivasan SR, Berenson GS. (1996).Persistent elevation of plasma insulin levels is associated with increased cardiovascular risk in children and young adults: the Bogalusa Heart Study. *Circulation* **93***:54*–9.

Barnett, A. H. (1991). Pathogenesis of diabetic microangiopathy: an overview. *Am J Med* **90** (Suppl 6A): 67-73.

Baynes, J.W. (1991). Role of oxidative stress in development diabetic complications. *Diabetes* **40**: 405-412.

Beckman JA, Creager MA, Libby P (2002): Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *JAMA*; **287**:2570– 2581.

Bennett PH. (1989) Classification of diabetes mellitus: theory and practice. Edited by: Ellenberg, Rifkin & Porte. Published by: Elsevier Science B. V, *Biomedical Division*. Vol. **1** 409-414.

Bhatia S and Shukla R. (2003) Antioxidant status, lipid peroxidation and nitric oxide end products in patients of type 2 diabetes mellitus with nephropathy. *Clinical Biochemistry*;**36**: 557-562.

Bonnefont-Rousselt D, Jaudon MC, Issad B, Cacoub P, Congy F, Jardel C, Delattre J, Jacobs C. (2000). Antioxidant status of elderly chronic renal patients treated by continuous ambulatory peritoneal dialysis. *Nephrol Dial Transplant* **12**: 1399-1405.

Brigelius-Flohe, R. (1999). Tissue specific function of individual glutathione peroxidases. *Free Rad Biol Med* **27**: 951-957.

Brophy W A, Sazon T, Forson R., (2007). Role of oxidative stress in diabetes complications. *Diabetes* **50***;* 309 – 410.

Burtis CA, Ashwood ER, Burns DA (2006) Carbohydrate In; Textbook of Clinical chemistry and molecular diagnostics Sacks DB edt, Chapter 25, **4th edition**. Saunders; p. 878-880.

Calisti L, and Tognetti S. (2005) Measure of glycosylated hemoglobin. *Acta Biomed*; **76(**3): 59-62.

Cavalca V, Cighetti G, Bamonti F, *et al*. (2001). Oxidative stress and homocy- steine in coronary artery disease. *Clin Chem*; **47** :887-92.

Ceriello A, Morocutti A, Mercuri F, Quagliaro L, Moro M, Damante G, Viberti GC. (1997) Defective intracellular antioxidant enzyme production in type I diabetic patients with nephropathy. Diabetes **49**: 2170-2177.

Dandona JS and Thorpe S R, (2008). Role of oxidative stress in diabetic complication: a new perspective on an old paradigm. *Diabetes* **48**: 1-9

D'Autréaux B, Toledano MB. (2007) ROS as signaling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol*. **8**:813–24.

Denham Harman A. Pathogenesis of type 2 diabetes: metabolic and molecular implications for identifying diabetes genes. Diabetes Rev 5: 177-269 (1956).

DeFronzo RA. (1997). Pathogenesis of type 2 diabetes: metabolic and molecular implications for identifying diabetes genes. *Diabetes Rev* **5**: 177-269.

Dincer Y, Alademir Z, Ilkova H, Akcay T. (2002); Susceptibility of glutatione and glutathione- related antioxidant activity to hydrogen peroxide in patients with type 2 diabetes: effect of glycemic control. *Clin Biochem* **35**: 297-301.

Dodu, S.R. (1958). The incidence of diabetes mellitus in Accra (Ghana), a study of 4,000 patients. *Afr. Med. J.*; **7**: 129-13.

Evans, J.L, Goldfine, I.D Maddux, B.A, and Grodsky, GM (2003). Are Oxidative Stress-Activated Signaling Pathways Mediators of Insulin Resistance and ß-Cell Dysfunction? *Diabetes*; **52**:1-8.

Facchini FS, Humphreys MH, DoNascimento CA, Abbasi F and Reaven GM. (2000). Relation between insulin resistance and plasma concentrations of lipid hydroperoxides, carotenoids, and tocopherols. *Am J Clin Nutr*; **72:**776-9.

Fikree M, Hanafi B. Hussain ZA, Masuali EM, (2006). Glycemic control of type 2 diabetes mellitus. *Bahrain Med Bull*; **28** (3); 1-6.

Ford ES. (2001). Vitamin supplement use and diabetes mellitus incidence among adults in the United States. *Am J Epidemiol 2001*; **153**:892-7.

Fowler, G.C and Vasudevan, D.A. (2010). Type 2 diabetes mellitus. Managing hemoglobin A1c and beyond. *Southern Med J*: **103** (9): 911 -16.

Freeman BA, Crapo JD (1982) . Biology of disease: free radicals and tissue injury. *LabInvest* ; **47**: 412 26.

Frei, B., Stocker, R, Ames, B.N.(1988) Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc Natl Acad Sci* U S A; **85**, 9748-52.

Fridovich I. (1995). Antioxidants and lipid peroxidation status in diabetic patients with and without complications. *Annu Rev Biochem***., 64,** 97-112**.**

Friedewald WT, Levy RI, Fredrickson DS. (1972) Estimation of the concentration of low- density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* **18**: 499-502.

Gallou G, Ruelland A, Legras B, Maugendre D, Allannic II, Cloarec L. (1993). Plasma malonialdehyde in type 1 and type 2 diabetic patients. *Clin Chim Acta* **214**: 227-234.
Gerschman R, Gilbert D. L., Nye S.W., Dwyer P., Fenn W.O. (1954) Oxygen poisoning and x-irradiation—A mechanism in common. *Science*; **119**, 623–626.

Gorogawa S, Kajimoto Y, Umayahara Y, Kaneto H, Watada H, Kuroda A, *et al*. (2002).Probucol preserves pancreatic beta-cell function through reduction of oxidative stress in type 2 diabetes. *Diabetes Res Clin Pract*; **57**: 1-10.

Griesmacher A, Kindhauser M, Andert SE, Schreiner W, Toma C, Knobel P, Pietschmann, Prager R, Schnack C, Schernthaner G, Mueller MM. (1995). Enahanced serum level of thiobarbituric acid reactive substances in diabetes mellitus. *Am J Med* **98**: 469- 476.

Grundy, S.M (2006). Metabolic syndrome: connecting and reconciling cardiovascular and diabetes worlds. *J Am coll cardiol*, **47**(6); 1093 – 1100.

Guerrero-Romero F and Rodriguez-Moran M. (2005). Complementary therapies for diabetes: the case for chromium, magnesium, and antioxidants. *Arch Med Res*; **36**: 250-7.

Gutteridge JMC. (1995). Lipid peroxidation and Antioxidants as Biomarkers of tissue damage. *Clin Chem* **41**: 1819-1828.

Halliwell B, Gutteridge JM. (2007).The measurement and mechanism of lipid peroxidation in biological systems. *Trends Biochem Sci*; **15**:129–35.

Halliwell B. (2000). Lipid peroxidation, antioxidants and cardiovascular disease: how should we move forward? *Cardiovasc Res*; **47:**410-8.

Horton AA and Fairhurst S. (1987). Lipid peroxidation and mechanisms of toxicity. *Crit Rev Toxicology*; **18**:27-79.

Hu FB, Manson JE, Stampfer MJ, Colditz G, Liu S, Solomon CG and Willett WC. (2001)Diet, lifestyle and the risk of type 2 diabetes mellitus in women. *NEngl J Med*; **345**:790-7

Imlay JA. (2003). Pathways of oxidative damage. *Annu Rev Microbiol.*; **57**:395–418.

Kalaivanam KN, Dharmalingam M, Marcus SR. (2006). Lipid peroxidation in type 2 diabetes mellitus. *Int J Diab Dev Ctries;* **26**: 320-21.

Kangralkar V. A., Shivraj D. Patil R. Bandivadekar M. (2010). *International Journal of Pharmaceutical Applications.* Vol1,Issue **1** pp 38 – 45.

Kedziora-Kornatowska KZ, Luciak M, Blaszczyk Pawlak W. (1998). Lipid peroxidation and activities of antioxidant enzymes in erythrocytes of patients with non-insulin dependent diabetes mellitus with or without nephrophathy. Nephrol Dial Transpant; **13:** 2829-32.

Kesavulu MM, Giri R, Kameswara RB, Apparao C. (2000). Lipid peroxidation and antioxidant enzyme levels in type 2 diabetics with microvascular complications. *Diabetes Metab;* **26**: 387-92.

King GL. (2008). The role of inflammatory cytokines in diabetes and its complications. *J Periodontol*; **79:** 1527-34. 22.

King H, Aubert R.E, Herman W.H. (2004). Global burden of diabetes 1995-2025; Prevalence, numerical estimates, and projection. *Diabetes Care*; **21**: 1414-1431.

Kiyosawa, H., Suko, M., Okudaira, H, Murata, K., Miyamoto, T., Chung, M.H., Kasai, H. & Nishimura, S. (1990) Cigarette smoking induces formation of 8-hydroxydeoxyguanosine, one of the oxidative DNA damages in human peripheral leukocytes. *Free Radic Res Commun*; **11**, 23-7.

Kuthan H, Recoba H. Barron C. Alvarez,(1986). A spectrophotometric assay for superoxide dismutase activities in crude tissue fractions. *Biochem J*. **237**: 175-80

Kuzuya T and Matsuda A. (1997) Classification of diabetes on the basis of aetiologies versus degree of insulin deficiency. *Diabetes Care* **21**: 666-667.

Laight DW, Carrier MJ and Anggard EE. (2000) Antioxidants, diabetes and endothelial dysfunction. *Cardiovasc Res*; 47:457-64.

Lamarche B, Despres JP, Moorjani S, Cantin B, Dagenais GR, Lupien PJ. (1996). Triglycerides and HDL-cholesterol as risk factors for ischemic heart disease. Results from the Quebec cardiovascular study. *Atherosclerosis*; **119**: 235-45.

Lee AY, Chung SK, Chung SS (1995) Demonstration that polyol accumulation is responsible for diabetic cataract by the use of transgenic mice expressing the aldose reductase gene in the lens. Proc Natl Acad Sci USA 92:2780–2784.

Liu S and Manson JE (2001): Dietary carbohydrates, physical inactivity, obesity, and the 'metabolic syndrome' as predictors of coronary heart disease. *Curr Opin Lipidol*, **12**:395-404.

Losada M, Alio JL. (1996) Malondialdehyde serum concentration in type 1 diabetic with and without retinopathy. *Doc Ophthalmol;* **93**: 223-9.

Mahboob M, Rahman RF, Grover P. (2005). Serum lipid peroxidation and antioxidant enzyme levels in male and female patients with diabetes. *Singapore Med J*. ; **46**: 322-324.

Maiese JD and McGarry JD. (2010). Metabolic derangements and treatment of diabetic kitoacidosis *N E JM*. **309**: 159.

Maritim AC, Sanders RA, Wattkins JB. (2003). Diabetes, oxidative stress and antioxidants, a review, *Journal of Biochemical and Molecular Toxicology*; **17**; 24 – 38.

Martin - Gallan CM, Young IS, Lightbody JH. (2003). Oxidative stress in heamodialysis. *QJ Med* **87**: 673-683.

Mbanya J.C.N, Riste L, Cruickshank J.K, Forhan A, Forrester T, Anderson N.M, Balkau B, Bennett F, Ngogang J.Y, Wilks R. (1999). Standardized comparison of glucose intolerance in West Africa—origin populations of rural and urban Cameroon, Jamaica, and Caribbean Migrants to Britain. *Diabetes Care*; **22:** 434–440.

McCord A and Fridovich M., Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes, *J. Biol. Chem*., 1969, **279(41)**, 42351–42354.

Mehra PD, Herod DA, Barnett J. (2007). Effects of vitamin E on susceptibility of LDL and LDL subfractions to oxdation and on protein glycation in NIDDM. *Diabetes Care* **18**: 807-8

Mittal CK and Murad F. (1977) Activation of guanylate cyclase by superoxide dismutase and hydroxyl radical: a physiological regulator of guanosine 3′,5′-monophosphate formation. *Proc Natl Acad Sci USA*; **74**:4360– 4364.

Mukherjee B, Anbazhagan S, Roy A, Ghosh R, Chatterjee M. (1998).Novel implications of the potential role of selenium on antioxidant status in streptozotocin-induced diabetic mice. *Biomed & Pharmacother* **52**: 89-95.

Nathan DM, Singer DE, Hurxthal K, Goodson JD. (1984). The Clinical information value of the glycosylated hemoglobin assay. *N Engl J Med* **310**; 341-46.

Nehru B and Kanwar SS. (2004) N-acetylcysteine exposure on lead-induced lipid peroxidative damage and oxidative defense system in brain regions of rats. *Biol Trace Elem Res*; **101**: 257-64.

Nurooz-Zadeh J, Rahimi A, Tajaddini-Sarmadi J, Tritchler H, Rosen P, Halliwell B, *et al.*, (1996). Relation- ships between plasma measures of oxidative stress and metabolic control in NIDDM. *Diabetologia;* **40**: 647-53.

Obrosova IG, Vanlteysen C. Fathallah L, Cao X, Greene DA, Stevens MJ (2002). An aldose reductase inhibitor reverses early diabetes –induced charges in peripheral nerve function, *FASEB J*. **16**; 123-125.

Ohtsuki T, Malasumoto M, Suzuki K, Taniguchi N, Kadanada T. (1995). Mitochondrial lipid peroxidation & superoxide dismutase in rat hy- pertensive target organs. *Am J Physiol*; **268**: 1418-21.

Pagila DE, Valentine WN. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *JLab Clin* Med 70: 158-169.

Pasaoglu H, Sancak B, Burkan N. (2004). Lipid peroxidation and resistance to oxidation in patients with type 2 diabetes mellitus. *Tohuku J Exp Med*; **203**:211-218.

Pasupathi P, Manivannari P, Uma M, Deep M, (2009). Glycated haemoglobin as a stable indicator of type 2 diabetes. *Int J Pharm Biochem Res*: **1**(2); 53-56.

Peerapatdit T, Patchanans N, Likidlilid A, Poldee S, Sriratanasathavorn C. (2006) Plasma lipid peroxidation and antioxidiant nutrients in type 2 diabetic patients. *J Med Assoc Thai*; **89** (Suppl 5): S147-55.

Poon HF, Calabrese V, Scapagnini G, Butterfield DA. (2004) Free radicals and brain aging. *Clin Geriatr Med*; **20**:329-59.

Rice-Evans CA. (1994). Formation of free radicals and mechanisms of action in normal biochemical processes and pathological states. In Rice-Evans CA, Burdon RH (Eds). Free radical damage and its control. *Elsevier Science B V*. **45:** 456-459.

Rosen P, Nawroth PP, King G, Moller W, Tritschler HJ and Packer L. (2001). The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO- MCBN, the American Diabetes Association and the German Diabetes Society. *Diabetes Metab Res Rev*; **17**:189-212.

Ruhe RC and McDonald RB (2000). Use of antioxidant nutrients in the prevention and treatment of type 2 diabetes. *J Am Coll Nut*r **20** (5 Suppl): 363S-9S.

Salonen JT, Nyyssonen K, Tuomainen TP, Maenpaa PH, Korpela H *et al.* (1995)Increased risk of non-insulin dependent diabetes at low plasma vitamin E concentrations: a four year follows up study in men. *BMJ;* **311**: 1124-7.

Satoh K. (1978). Serum lipid peroxides in cerebro-vascular disorder. *Clin Chim Acta*; **28**: 37-43.

Saxane A. (1993). Type 1 (Insulin-dependent) diabetes mellitus: etiology, pathogenesis, and natural history. In DeGroot JL & Jameson LJ (eds): *Endocrinology, Ed* 4. Vol **1**, Philadelphia, WB Saunders, 763-775.

Semenza GL. (2007) Life with oxygen. Science. 2007; 318:62–4. 155. Sies, H. Strategies of antioxidant defense. *Eur J Biochem*; **215**; 213-9.

Shaw JE, Sicree RA, Zimmet PZ. (2010). Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice*; **87**: 4-14.

Sies P. (2000) Protection of insulin-producing RINm5F cell against cytokine-mediated toxicity through overexpression of antioxidant enzymes. *Diabetes* **49**: 1123-1130.

Slater TF. (1984) Free-radical mechanisms in tissue injury. *Biochem J*; 222: 1-15. 7.

Soliman GZ. (2008). Blood lipid peroxidation (superoxide dismutase, malondialdehyde, glutathione) levels in Egyptian type 2 diabetic patients. *Singapore Med J*; **49**: 129-36.

Sozmen EY, Sozmen B, Delen Y, Onat T. (2001). Catalase/ superoxide dismutase (SOD) and catalase/ paraoxonase (PON) ratios may implicate poor glycemic control. *Arch Med Res*; **32**: 283-7.

Stefanovic JK, Prakash M, Ibrahim MS. (2010). Relationship between free iron and glycated hemoglobin in uncontrolled Type 2 diabetic patients associated with complications. *IJCB*: **23** (1); 67-70.

Su Y, Liu XM, Sun YM, Jin HB, Fu R, Wang YY, *et al.*(2008) The relationship between endothelial dysfunction and oxidative stress in diabetes and prediabetes. *Int J Clin Pract*; **62:** 877-82.

Sundaram RK, BhaskarA, Vijayalingam S, Visvanatthan M, Mohan R, Sanmugasundaram KR. (1996). Antioxidant status and lipid peroxidation with and without complications. *Clin Sci* **90**: 255-260.

Symons MCR and Gutteridge JMC (1998) (Eds). Free radicals and iron: Chemisrey. Biology and medicine. Oxford Uni Press UK.

Tare K, Limbu YR, Rai SK, Kurokawa M, Yanagida J, Rai G, (2007). The prevalence of type 2 diabetic mellitus and impaired fasting glucose in semi-urban population of Nepal. *Nepal Med Coll J*; **9**: 154-156.

Targher M, Bozikov V, Bozikov J, Meelko Z, Jandric I, Romic Z. (2004) Impact of Glycemic control on antioxidant enzyme acivity in patients with type 2 diabetes mellitus. *Diabetologia Croatica*.; **33**: 131-135.

Tinder P (1969). Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Ann Clin Biochem* **6:**2 4-27.

Van der Jagt DJ, Harrison JM, Ratliff DM, Hunsaker LA, Vander Jagt DL. (2001) Oxidative stress indices in IDDM subjects with and without long-term diabetic complications. *Clin Biochem*; **34**: 265-70.

Varvarovska J, Racek J, Stozicky F, Soucek J, Trefil L, Pomachacova R. (2003). Parameters of oxidative stress in children with type 1 diabetes mellitus and their relatives *Journal of Diabetes and its Complications* **17**: 7-10

Veerma M, Paneri S, Badi P, Roman PG. (2006). Effect of increasing duration of diabetes mellitus on glycated hemoglobin and insulin sensitivity. *Indian J Clin Biochem*: **21**(1); 142-46.

Van PJ, Patne AB, Fawade MM. (2003) Assessment of plasma antioxidant levels in type 2 diabetes patients. *Int J Med res*. **3**: 1796-1800.

Winterbourn CC. (2008). Reconciling the chemistry and biology of reactive oxygen species. *Nat Chem Biol*.; **4**:27886.

Wolff SP. (1993) Diabetes mellitus and free radicals. Free radicals, transition metals and oxidative stress in the aetiology of diabetes mellitus and complications. *Br Med Bull* **49**: 642-652.

World Health Organization (WHO). (2000). Diabetes Mellitus: Report of a WHO Study Group. Geneva: WHO, *Technical Report Series* 727.

World Health Organisation (WHO) Repot Part 1: Diagnosis and classification of diabetes mellitus WHO, Geneva (1999).

World Health Organization (WHO). (2000). Diabetes Mellitus: Report of a WHO Study Group. Geneva: WHO, *Technical Report Series* 727.

Yokoyama H, Jensen JS, Myrup B, Mathiesen ER, Ronn B, Decker T. (1990). Raised serum sialic acid concetration precedes onset of microalbuminuria in IDDM. A 10-year follow up study. *Diabetes Care* **19**: 435-440.

Young IS, Torney JJ, Trimble ER. (1991). The effect of ascorbate supplementation on oxidative stress in the streptocin diabetic rat. *Free radical Biology and Medicine* **8**; 752-758.

Zimmet P Unwin N, Shaw J, Alberti KG. (2002). Impaired glucose tolerance and impaired fasting glycaemia: the current status on definition and intervention. *Diabet Med*. **19**(9):708-23.

APPENDICES

APPENDIX I INFORMED CONSENT FORM FOR THE STUDY

STUDY TITLE: LIPID PEROXIDATION AND ANTI-OXIDANT STATUS IN TYPE 2 DIABETES MELLITUS IN GHANA.

Dear Volunteer,

I kindly ask your permission to enter you into a study which I will proceed to describe. In order to ensure that you are informed about being in this research, we are asking you to read (or have read to you) this consent form. You will also be asked to sign it (or make your mark). We will give you a copy of this form. This consent form might contain some words that are unfamiliar to you. Please ask us to explain anything you may not understand.

Why this study is planned

You have been asked to participate in the above study in order to help determine oxidative stress and antioxidants status in type 2 diabetes. The major contributor to the increasing number of diabetic patients is type 2 diabetes. Type 2 diabetes is characterized by inability to utilize glucose in the presence of insulin. Diabetes lead to depletion of cellular antioxidant defense system and increased levels reactive oxygen species. The imbalance between the antioxidant defense system and reactive oxygen species leads to oxidative stress in diabetes. Oxidative stress is an important trigger in the onset and progression of diabetes and its complications. This may offer a unique therapeutic option for diabetes and its complications by using antioxidants or nutrients with high antioxidant capacity. Antioxidants have been shown to reduce indices of oxidative stress measures. So far their possible therapeutic role has been underestimated in diabetes research. They are safe and inexpensive in comparison to other therapies.

General Information and your part in the study

We will take 6 ml (about two teaspoonfuls) venous blood from you for routine laboratory analysis.

Possible Benefits

Your participation may help determine the role oxidative stress and antioxidants play in diabetes and its complication. And this will help suggest alternatives therapy for type 2 diabetes in the form of antioxidants.

Possible Risks

The amount of blood collected is harmless, although there may be a slight pain at the bleeding site. Sterile techniques and disposable, single-use equipment will be used at all times.

Withdrawal from study

This study is strictly voluntary. Should you, at any point during the study, decide that you do not wish to participate any further, you are free to terminate the participation. Your decision will not affect the health care you would normally receive.

Confidentiality

All information gathered would be treated in strict confidentiality. When results of this study are reported in medical journals or at medical meetings, identities will be kept anonymous. All medical records will be stored by the researchers in safe cabinets.

Contacts

If you have any questions about this study or study-related problems, you may contact Dr. Asare Anane Henry **(Tel: 024 6024002)** and Dr. Amanquah Seth **(024 4293987) of the Chemical Pathology Department**, Korle-Bu Teaching hospital. You are free to ask any questions.Thank you.

Participant: I understand all the above and hereby agree to enter into this study

……..
……

Name of participant Date

.....
.....

Signature or Thumb print of Participant

……
..
……
..

Signature of Witness

…….
……

Name of Investigator Date

……
……

Signature of Investigator

APPENDIX II QUESTIONNAIRE OF THE STUDY

STUDY TITLE: LIPID PEROXIDATION AND ANTI-OXIDANT STATUS IN TYPE 2 DIABETES MELLITUS SUBJECTS IN GHANA.

Name (surname in blocks)

Date: Participant ID:

PERSONAL DETAILS

SEX: MALE ☐ FEMALE ☐

DATE OF BIRTH: …… OR APPROXIMATE AGE: ……

Are you currently pregnant? Yes☐ No ☐

Height (m) …… Weight (Kg): ……..…. BMI: …….. BP: ……

LIFE STYLE

Smoking:
Smoker: has smoked cigarettes regularly for at least 6 months ☐

Ex-smoker: stopped for at least 1 year after regular smoking ☐

Never ☐

Drinking:

Drink				
	Never	Once a while	<3/week	>3/week

Beer				
Palm wine				
Whisky/Gin				
Local liquor				

F. MEDICAL HISTORY
Has a doctor ever told you that you have any of the following conditions?

(If yes please specify date of diagnosis)

Diabetes Yes ☐ No ☐

High blood pressure (doctor gave pills): Yes ☐ No ☐

Do have any disease apart from the diabetes? Yes/No

If yes what kind of disease is it?

MEDICATIONS:
List any medications that you are currently taking for diabetes

Have you ever taken any antioxidant supplements for at least the past month?

Yes ☐ No ☐

Yes ☐ No ☐

(If yes please specify): ……
……
……

DIETARY PATTERN

How many times do you usually eat in a day?
Once a day ☐ 2 times a day ☐ 3 times a day ☐ >3 times a day ☐

Meals usually prepared at home

Breakfast ☐ Lunch ☐ Supper ☐