# SERUM GAMMA-GLUTAMYL TRANSFERASE, TOTAL ANTIOXIDANT CONCENTRATION AND HISTOLOGICAL CHANGES IN LIVER, KIDNEY AND HEART OF ALLOXAN-INDUCED DIABETIC RATS.

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**DEDICATED**

TO

My

HUSBAND

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## ABSTRACT

This study was performed to determine gamma-glutamyl transferase(GGT) and total antioxidant capacity(TAC) and examine the liver, kidney, heart changes in alloxan-induced diabetes. 40 wistar rats of both sexes weighing between 160g-210g were divided into two groups A (control) and B (diabetic) consisting of 20 rats each. Group A received normal saline while group B were given a single dose of alloxan monohydrate at 150mg/kg body weight intraperitoneally. On 3rd and 5th weeks respectively, 10 rats from each group were sacrificed, blood samples collected and organs excised for biochemical and histological studies. The mean serum GGT concentration of the diabetic group at 3 and 5 weeks (4.0±0.2iu/l and 4.4±0.2iu/l respectively) were significantly higher ($p<0.001$) when compared to the control group (0.8±0.2iu/l and 0.8±0.1iu/l) at 3 and 5 weeks respectively while the mean serum TAC of the diabetic group at 3 and 5 weeks (0.8±0.1mmol/l and 0.6±0.1mmol/l) respectively were significantly lower ($p<0.001$) when compared to the control (1.6±0.1mmol/l and 1.7±0.1mmol/l) at 3 and 5 weeks respectively. There was a significant negative correlation ($p<0.001$) between GGT and TAC in the experimental groups ($r = -0.707$ and $r = -0.897$) at 3 and 5 weeks respectively. There was a significant increase in the body weights of the control group while the diabetic group showed a significant decrease in their body weights ($p<0.05$).

The mean Urea, Creatinine, ALT, AST, TC, LDL-C, VLDL-C, TG, FPG and HbA1c values were significantly increased ($p<0.001$) in the diabetic group when compared to the control group while the mean serum HDL-C value of the diabetic group decreased significantly ($p<0.001$) when compared to the control at 3 and 5 weeks of the study. From this study, persistent hyperglycaemia resulted in a number of biochemical and pathological abnormalities in the diabetic rats while the non-diabetic rats were normoglycaemic and showed no biochemical or pathological changes. The study suggests that diabetes results to oxidative stress with GGT and TAC serving as reliable markers, and the pathological and biochemical changes in liver, kidney and heart may be regarded as complications of diabetes in these organs.

# CHAPTER ONE

# INTRODUCTION

Diabetes mellitus often simply referred to as diabetes is one of the most important health problems with very high prevalence, morbidity and mortality. It is a group of metabolic diseases characterized by chronic hyperglycaemia resulting from defects in insulin secretion, action or both (American Diabetes Association ADA, 2004).

Insulin is a hormone produced by the pancreas that allows glucose from food to enter the bodyøs cells where it is converted into energy needed for muscles and tissues to function. As a result, a person with diabetes does not absorb glucose properly, and glucose stays circulating in the blood (hyperglycaemia) damaging tissues over time. This damage leads to life threatening health complications (International Diabetes Federation IDF Diabetes Atlas, 2011).

Diabetes is the worldøs largest endocrine disorder with deranged carbohydrate, fat and protein metabolism (Ghaisas, 2009). The World Health Organization in 2002 predicted that the worldwide number of patients with diabetes will double by the year 2025, from the current number of approximately 150 million to 300 million.

More recently in 2010, it was estimated that around 285 million people were diabetic, and this number has been predicted to reach 438 million by the year 2030, accounting for the population aged 20-79 (Shaw *et al,* 2010).

Uncontrolled or poorly controlled diabetes increases both short and long term complications like micro- and macrovascular complications, the most common complications are atherosclerosis, nerve damage, renal failure, blindness, infertility and so on. The liver has also been shown to be affected by diabetes, although the exact mechanism still remains unclear (Gavin *et al,* 1999).

Alloxan is a diabetogenic agent used to induce experimental model of diabetes. Administration of alloxan to different animals produces necrosis of the islets, several features common to those observed in human diabetes (Quan *et al*, 2001).

Animal models of diabetes are increasingly being used in the investigation of the pathogenesis of diabetes and long term diabetic complications seen in clinical studies (Bell and Hye, 1983).

Diabetes mellitus results in multi-system consequences which present with biochemical and anatomical changes. Disturbances in the metabolism of carbohydrates, proteins and fats are the biochemical consequences while micro- and macrovascular complications are the anatomical derangements.

Increasing evidence in both experimental and clinical studies suggest that oxidative stress plays a role in the pathogenesis of diabetes mellitus, and the free radicals formed as a result leads to a decline in the anti-oxidant defense mechanism which consequently lead to increased risk and development of complications of diabetes, all of which affect longevity and quality of life.

Moreover, accumulating evidence suggests that oxidative stress plays a pivotal role in the aetiology of diabetic complications.

Many biochemical pathways associated with hyperglycaemia increase the production of free radicals leading to oxidative stress, including auto-oxidation, the polyol pathway, prostanoids synthesis, protein glycation and the protein kinase C (PKC) pathways (Guigliano *et al*, 1996).

Increased oxidative stress is a widely accepted participant in the development and progression of diabetes and its complications (Baynes, 1991; Baynes and Thorpe, 1999; Ceriello, 2000).

ϒ- glutamyl transferase (GGT) has long been used as a liver function test and a marker of excessive alcohol intake, but recently, it has been reported that GGT is closely related to oxidative stress, and that its activity is associated with diabetes mellitus, inflammation, cardiovascular diseases, stroke and mortality in the general population (Whitfield, 2001).

In diabetes, persistent hyperglycemia causes increased production of free radicals or Reactive Oxygen Species (ROS) as a result of glucose oxidation, lipid peroxidation and non-enzymatic glycation of proteins which contributes to the development of complications.

Living organisms have developed complex antioxidant systems to counteract reactive species and to reduce their damage. These antioxidant systems include enzymes such as catalase, superoxide dismutase and glutathione peroxidase; macromolecules such as albumin, ceruroplasmin and ferritin, and an array of small molecules, including ascorbic acid, -tocopherol, -carotene, ubiquinol-10, reduced glutathione (GSH) (Ames *et al,* 1993) methionine, uric acid and bilirubin (Yu, 1994).

The Total Antioxidant Concentration (TAC) serves as a general marker of the antioxidant defenses.

In vitro and clinical studies may provide additional useful ways to probe the inter connections of oxidative stress and diabetes, and there is a need to continue to explore the mechanisms by which increased oxidative stress accelerates the development of complications in diabetes (Maritim *et al*, 2003).

## 1.1 Justification of the Study

Prospective studies have described that high levels of GGT enzyme is associated with subsequent development of diabetes (Lee *et al*, 2003; Nakanishi *et al*, 2003; Nakanishi *et al*, 2004). Since oxidative stress appears to be a key component of many reactions associated with complications of diabetes, it will be of interest to study the relationship between GGT and TAC in diabetes and its possible complications.

Previous studies have reported liver, kidney and heart damages from diabetes resulting in hepatopathy, nephropathy and cardiovascular problems respectively, but there is still need to examine the pathological changes in these organs in relation to their biochemical markers.

## 1.2 Significance of Study

Predicting an increased risk of developing diabetes and its related complications as well as knowledge of the anti-oxidant defense status will encourage an individual make lifestyle changes or obtain treatment leading to a decreased risk. Additionally, the quality of life may be improved, thereby decreasing the overall burden on the healthcare systems.

## 1.3 Aims and Objectives

**i.** To determine the serum levels of GGT and TAC in diabetic rats.

**ii.** To assess the association between GGT and TAC in diabetes.

**iii.** To examine the effect of diabetes on the histology of the liver, kidney and heart using their biomarkers.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 History of Diabetes

Diabetes was one the first diseases described (Ripoll *et al*, 2011). For 2000 years, diabetes has been recognized as a devastating and deadly disease (Melissa, 2008).

The term diabetes was coined by Aretaeus the Cappadocian, a Greek physician in the second century AD who named the condition diabainein which is a Greek word meaning ÷to pass throughø. He described patients who were passing too much water (polyuria) like a siphon.

In 1675, Thomas Willis added mellitus to the word õdiabetesö when he noticed that the urine of people with diabetes had a sweet taste (glycosuria). This sweet taste has been noticed in urine by the ancient Greeks, Chinese, Egyptians, Indians and Persian (News-Medical.Net, 2012). Mellitus comes from a Latin word ÷melø which means õhoneyö.

In 1776, Matthew Dobson confirmed that the sweet taste was because of an excess of a kind of sugar in the urine and blood of people with diabetes (News-Medical.Net, 2012).

The ancient Indians tested for diabetes by observing whether ants were attracted to a personøs urine and called the ailment õSweet Urine Diseaseö (Madhumeha). The Korean, Chinese and Japanese words for diabetes are based on the same ideograph which means ÷Sugar Urine Diseaseø (News-Medical.Net, 2012).

## 2.2 Epidemiology

A global estimate in 2010 showed that about 285 million people had diabetes, with type 2 having a greater per centage. Its incidence is increasing rapidly and by 2030, this number is estimated to almost double (Wild *et al*, 2004).

The International Diabetes Federation IDF in 2011 stated that about 4.6 million people between 20-79 years of age died from diabetes in 2011, accounting for 8.2 per cent of the global cause of mortality in people of this age group.

The increased incidence in developing countries follows the trend of urbanization and lifestyle changes, perhaps, most importantly a õwestern styleö diet. This has suggested an environmental (that is, dietary) effect, but there is little understanding of the mechanism(s) at present, though there is much speculations, some of it most compellingly presented (Wild *et al*, 2004).

IDF estimates that as many as 183 million people worldwide, or half of those with diabetes are not aware of their conditions. No country has diagnosed every person that has diabetes. In Sub-Saharan Africa, where resources are often lacking and governments may not prioritize screening for diabetes, the proportion of people with undiagnosed diabetes can reach up to 90 per cent in some countries (Evaristo-Neto *et al*, 2010).

Moreover, in high income countries, about one-third of people with diabetes have not been diagnosed. Globally, 85 per cent of all people who remain undiagnosed are in low and middle income countries (IDF Diabetes Atlas, 2011).

In 2011, the IDF stated that diabetes has been traditionally viewed as a disease of rich countries. However, estimates of diabetes prevalence showed that four out of five people with diabetes live in countries classified by the World Bank as low and middle income countries. Of the 3.6 billion adults living in low, middle and high income countries in 2011, 291 million have diabetes (World Bank, 2011) compared to 75 million adults with diabetes living in high-income countries.

## 2.3 Blood Glucose

A type of sugar present in the blood when absorbed from the intestinal tract, glucose is transported via the portal vein to the cells that need it while excess is stored in the liver as glycogen, which serves as a reservoir to replenish blood glucose when the level falls. The storage of glycogen in the liver is enhanced by the hormone, insulin.

In addition, insulin helps bodyøs cells utilize glucose, it is produced by the  -cells in the pancreatic islets and released into the blood when the glucose level rises especially after

meal. However, the glycogen storage is limited because of the bulky nature of the glycogen molecule.

Glucose uptake by the cells is mediated by membrane transporters called Glucose Transporters GLUT. Glucose that is taken up by a cell may be oxidized to pyruvate in the cytosol, and electrons generated from these processes are transferred to the mitochondria. Pyruvate generated from the Embden-Meyerhofføs pathway is oxidized to acetyl CoA in the mitochondria, which in turn undergoes further oxidation in the citric acid cycle.

## 2.4 Blood Glucose Regulation

Blood glucose regulation involves the maintenance of glucose in blood within the normal range, this can also be referred to as glucose homeostasis.

Glucose molecules are delivered to cells by the circulating blood and therefore, to ensure a constant supply of glucose to cells, it is essential that blood glucose levels be maintained at relatively constant levels. Level constancy is accomplished primarily through negative feedback systems, which ensure that blood glucose concentration is maintained within the normal range of 70 to 110 milligrams (0.0024 to 0.0038 ounces) of glucose per decilitre (approximately one-fifth of a pint) of blood (Norman, 2011).

Negative feedback systems work in such a way that when they sense changes in normal conditions in the body, they activate mechanisms that reverse these changes to normal. Negative feedback systems are critically important in homeostasis, the maintenance of relatively constant internal conditions. Disruptions in homeostasis lead to potentially life-threatening situations. The maintenance of relatively constant blood glucose levels is essential for the health of cells and thus the health of the entire body (Norman, 2011).

Several factors influence the regulation of blood glucose, these factors include hormonal and non hormonal which are discussed below.

### 2.4.1 Hormones involved in Blood Glucose Regulation

Blood glucose homeostasis is achieved by balancing the pancreatic endocrine hormones; insulin and glucagon. Several other hormones also influence blood glucose level by either inhibiting or enhancing the actions of the two pancreatic hormones mentioned earlier.

The table overleaf summarizes the effects of the listed hormones on blood glucose level.

**Table 2.1: Hormones of Blood Glucose Regulation**

| HORMONE | SOURCE | EFFECT | FUNCTIONS |
|---|---|---|---|
| Insulin | -cells of pancreas | Lowers blood glucose | - Promotes storage of glucose as glycogen<br>- Promotes glucose entry into cells.<br>- Promotes proteins and fatty acid synthesis. |
| Glucagon | -cells of pancreas | Raises blood glucose | - Promotes glycogenolysis.<br>- Promotes gluconeogenesis. |
| Somatostatin | -cells of pancreas | Lowers blood glucose | - Suppresses glucagon release from cells<br>- Suppresses release of insulin, pituitary tropic hormones.<br>- Reduces gut motility and further absorption of nutrients. |
| Growth Hormone | Anterior pituitary | Raises blood glucose | - Opposes the action of insulin. |
| ACTH | Anterior pituitary | Raises blood glucose | - Enhances cortisol release<br>- Promotes fatty acid release from adipose tissues. |
| Thyroxine | Thyroid gland | Raises blood glucose | - Promotes intestinal glucose absorption.<br>- Enhances glycogenolysis. |
| Cortisol | Adrenal Cortex | Raises blood glucose | - Opposes insulin action.<br>- Promotes gluconeogenesis. |
| Epinephrine | Adrenal Medulla | Raises blood glucose | - Enhances glycogenolysis.<br>- Promotes fatty acid release from adipose tissues. |

### 2.4.2 Non-Hormonal Factors involved in Blood Glucose Regulation

The non-hormonal factors that affect blood glucose concentration are listed below;

a. **Autonomic Nervous system:** this controls the breakdown of liver glycogen. It consists of two divisions; the Sympathetic and Parasympathetic. The former enhances breakdown of glycogen thereby increasing blood glucose level while the latter enhances glycogen synthesis and storage. The opposing actions of these two divisions results to the maintenance of blood glucose at certain concentration.

b. **Diet:** depending on the diet constituents, blood glucose level rises after meal, this rise is taken care of by the regulatory mechanism.

c. **Severe injury, Trauma and Infections:** these may lead to a release of inflammatory cytokines (TNF-  ) which oppose insulin release or action on peripheral tissues.

d. **Others** include; Pregnancy, Lifestyle and so on.

### 2.4.3 Regulation by Insulin Responsive Tissues

The maintenance of blood glucose homeostasis also requires the insulin target cells to effectively respond to the hormone insulin.

These peripheral tissues which include; liver, skeletal muscle and adipose tissue are the main insulin responsive or target tissues in the body.

When the sensitivity of the peripheral tissues is compromised such that they can no longer respond appropriately to insulin, a condition called ÷insulin resistanceø occurs leading to glucose accumulation in the blood (Dwi, 2012).

a. **Liver:** the liver plays a vital role in glucose homeostasis, and therefore any disruption in the equilibrium of the liver will affect the glucose balance in blood. Furthermore, the central and crucial role in the regulation of carbohydrate and glucose homeostasis offers a clue to the pathogenesis of glucose intolerance in liver diseases. The normal functioning of the liver

therefore is essential for the maintenance of blood glucose levels and of continued supply to organs that require a glucose energy source (Gavin *et al*, 1999).

b. **Adipose tissue:** the adipose tissue is another crucial regulator of glucose metabolism. An excessive abnormal amount of adipose tissue had been associated with the pathogenesis of diabetes. It is thus believed that imbalance in glucose homeostasis partially relies on the insensitivity or resistance of the adipose tissue to the action of insulin (Dwi, 2012).

c. **Skeletal muscle:** the skeletal muscle accounts for approximately 75 per cent of insulin-stimulated glucose uptake, serving as the primary site for postprandial glucose uptake. Insulin resistance in the skeletal muscle is also a hallmark of early onset of type 2 diabetes (Dwi, 2012).

## 2.5 Mechanism of Blood Glucose Regulation

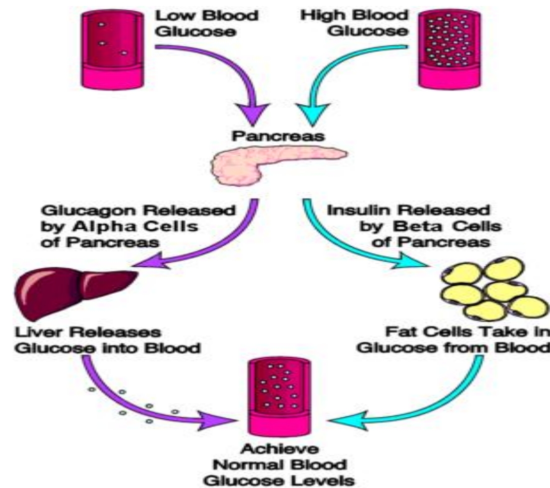
When blood glucose level falls to dangerous levels, for example in prolonged fasting state or excessive physical activity, glucagon is released from the   cells of the pancreas which stimulates glycogenolysis, releasing glucose from hepatocytes into the blood stream thereby raising blood glucose levels to normal.

As stated earlier, blood glucose level is principally controlled by the pancreatic islets. When blood glucose level rises, insulin is released from the   cells islet of langerhans which stimulates the liver to store excess glucose as glycogen (glycogenesis) and the muscle and adipose tissue cells to take up glucose from blood via GLUT 4 transporters which results in decrease in blood glucose level.

In addition, when insulin binds to the receptors on the cell surface, vesicles containing the GLUT 4 transporters come to the plasma membrane and fuse together by the process of exocytosis, thus enabling a facilitated diffusion of glucose into the cell. As soon as the glucose enters the cell, it is phosphorylated into Glucose-6-Phosphate in order to preserve the

concentration gradient so glucose will continue to enter the cell. Insulin also provides signals to several other body systems, and is the chief metabolic control in humans.

**Figure 2.1: Blood Glucose Regulation**




*By Norman James*

## 2.6 Impaired Blood Glucose Regulation

This refers to a derangement in the regulation of blood glucose levels. It is an intermediate state of carbohydrate metabolism between normoglycaemia and diabetes which implies that blood glucose levels are higher than normal but not high enough for a diabetes diagnosis. It can also be referred to as Pre-diabetes. The fasting and two hour post-prandial glucose values in normoglycaemia are <6.1 mmol/l (<110 mg/dl) and <7.8 mmol/l (<140mg/dl) respectively while in diabetes mellitus, the values are ×7.0 mmol/l (×126 mg/dl) and ×11.1 mmol/l (×200 mg/dl) respectively .

The two forms of this derangement or pre-diabetes states are discussed below.

a. **Impaired Fasting Glucose IFG:** refers to a high blood glucose level after an overnight fast. People with IFG have increased fasting blood or plasma glucose but are usually normoglycaemic after food consumption. It is a major risk factor for the development of diabetes and cardiovascular disease CVD. The fasting and two hour post-prandial glucose

values are ×6.1 - <7.0 mmol/l (×110 ó <126 mg/dl) and <7.8 mmol/l (<140mg/dl) respectively.

b. **Impaired Glucose Tolerance IGT:** refers to a high blood glucose level after a large glucose load or meal. IGT is also a risk factor for progression to full blown diabetes mellitus as well as CVD (Santaguida *et* al, 2008). The fasting and two hour post-prandial glucose values are <7.0 mmol/l (<126 mg/dl) and ×7.8 mmol/l (×140mg/dl) respectively.

Type 2 diabetes and IGT are also associated with obesity, aging and insulin resistance. However, not all with IGT will develop Type 2 diabetes.

## 2.7 The Insulin Hormone

Insulin is a hormone produced by the  cells of the islets of langerhans in pancreas which takes part in gluco-regulation. It is a small protein with a molecular weight of about 6000 Daltons, composed of two peptide chains A and B which are held together by two disulfide bonds with an additional disulfide bond formed within the A chain.

Insulin plays a key role in the control of intermediary metabolism which include carbohydrate, lipid, protein and mineral metabolism. Therefore, any derangements in insulin secretion have widespread and devastating effects on many organs and tissues.

### 2.7.1 Functions of Insulin

Insulin have diverse functions, these are listed below.

**a.** Regulation of glucose metabolism: dietary carbohydrate such as starch or sucrose is hydrolyzed in the small intestine to yield glucose which is then absorbed into the blood. Increased blood glucose concentrations trigger insulin release which stimulates the uptake, utilization and storage of glucose by the cells in the body. The effect of insulin on glucose metabolism depends on the target tissue.

**b.** Insulin facilitates the entrance of glucose into the liver, muscle and adipose tissue.

**c.** Insulin stimulates the hepatocytes to store glucose in the form of glycogen.

**d.** Regulation of lipid metabolism: because of the inter-relationship between the metabolic pathways for utilization of fats and carbohydrates, and considering insulin's profound effects on carbohydrate metabolism, it stands to reason that insulin also has important effects on lipid metabolism as stated below.

Insulin promotes lipogenesis in the liver; when the liverøs glycogen storage is saturated, excess glucose is taken up by the hepatocytes and shunted into fatty acid synthetic pathway which are then exported from the liver as lipoproteins, and converted to free fatty acids for use in other tissues viz adipocytes which in turn use them to synthesize triglycerides.

Insulin inhibits lipolysis in the adipose tissue by inhibiting the intracelleular lipase which hydrolyses triglycerides to release fatty acids. In the presence of insulin, glucose is taken up by the adipocytes which can be used in glycerol synthesis. Glycerol combines with the fatty acids from the liver to form triglycerides in the adipocytes.

**e.** Other functions: It promotes protein synthesis in the liver and muscle cells, enhances amino acid transport into cells, modulates DNA synthesis and transcription and controls arterial wall tension throughout the body. It stimulates cell growth and replication, activates Na-K ATPase in many cells causing an influx of potassium in the cells and also increases cell permeability to magnesium, potassium and phosphate ions.

### 2.7.2 Insulin Synthesis

Insulin is synthesized as a pre-prohormone or pre-proinsulin only in the beta cells of the pancreas. In the cisternae of the endoplasmic reticulum, its signal peptide is removed to form proinsulin which when exposed to several specific endo-peptidases yields the mature form of insulin which is then packaged into the secretory vesicles of the golgi within the cytoplasm. Upon stimulation the beta cells, insulin is secreted from the cell by exocytosis and diffuses into islet capillary blood.

Apart from the insulin produced in a normal individual in response to high blood glucose level, spurts of insulin are also produced throughout the day and night to ensure glycemic control even in resting periods. In other words, insulin production counters the joint actions of the numerous hyperglycaemic generating hormones.

### 2.7.3 Insulin Regulation

Insulin action is carefully regulated in response to circulating glucose concentrations. Insulin is not secreted if the blood glucose concentration is Ö3.3mmol/l, but is secreted in increasing amounts as glucose concentrations increase beyond this threshold (Gerich, 1993).

Postprandially, the secretion of insulin occurs in two phases: an initial rapid release of preformed insulin, followed by increased insulin synthesis and release in response to blood glucose. Long-term release of insulin occurs if glucose concentrations remain high (Cryer, 1992; Gerich, 1993).

There are many factors that regulate the amount of insulin in blood in order to achieve glycemic control. Before now, glucoregulation was thought to be bi-hormonal (insulin and glucagon) but it has come to lime light that both pancreatic and incretin hormones are involved (multi-hormonal).

These other hormones are discussed in this section under insulin agonists and antagonists.

#### 2.7.3.1 Insulin Agonists

These are hormones that imitate or enhance the glucoregulatory actions of insulin.

a. **Amylin:** a second beta cell hormone whose role complements that of insulin is isolated from pancreatic amyloid deposits in the islets of Langerhans. It is a 37óamino acid peptide, a neuroendocrine hormone co-expressed and co-secreted with insulin by pancreatic -cells in response to nutrient stimuli (Koda *et al*, 1992; More *et al*, 1991; Ogawa *et al*, 1990).

Preclinical findings indicate that amylin works with insulin to help coordinate the rate of glucose appearance and disappearance in the circulation, thereby preventing an abnormal rise in glucose concentrations (Pehling *et al*, 1984).

Amylin works to regulate the rate of glucose appearance from both endogenous (liver-derived) and exogenous (meal-derived) sources, and insulin regulates the rate of glucose disappearance (Bruse *et al*, 2002).

b. **Incretin hormones (GLP-1** and **GIP):** the name ÷incretinø is derived from ÷incretin effectø which explains signals from gut on food ingestion are important in the hormonal regulation of glucose. These hormones are from the proximal gut (gut peptides) and from the L-cells of the intestine.

**GLP-1:** Glucagon-like Peptide 1 stimulates insulin secretion when plasma glucose concentrations are high. It is derived from the pro-glucagon molecule in the intestine, GLP-1 is synthesized and secreted by the L-cells found mainly in the ileum and colon.

In the pancreas, GLP-1 stimulates insulin secretion in a glucose-dependent manner while inhibiting glucagon secretion (Nauck *et al*, 1997; Perfetti *et al*, 2000).

This hormone has a plasma half-life of about two minutes, and its disappearance is mainly regulated by dipeptidyl peptidase-IV (DPP-IV) enzyme, which cleaves and inactivates it. In addition, there have been reported observations of GLP-1 improving insulin sensitivity and enhancing glucose disposal (Zander *et al*, 2002).

**GIP:** Glucose-dependent Insulinotropic Peptide stimulates insulin secretion and regulates fat metabolism, but does not inhibit glucagon secretion or gastric emptying (Yip *et al*, 2000).

While GIP is a more potent incretin hormone, GLP-1 is secreted in greater concentrations and is more physiologically relevant in humans (Nauck *et al*, 1993).

GLP-1 and GIP are effectively stimulated by ingestion of a mixed meal or meals enriched with fats and carbohydrates (Hermann *et al*, 1995).

#### 2.7.3.2 Insulin Antagonists

These hormones act by counteracting or opposing insulin effects, and are listed below.

a. **Glucagon:** a key catabolic hormone with 29 amino acids, and secreted from alpha ( ) cells of the pancreas. It plays a major role in sustaining plasma glucose during fasting conditions by stimulating hepatic glucose production.

   Hepatic glucose production, which is primarily regulated by glucagon, maintains basal blood glucose concentrations within a normal range during the fasting state. When plasma glucose falls below normal, glucagon secretion increases, and results in hepatic glucose production and return of plasma glucose to the normal range (Gereich *et al*, 1979; Orci *et al*, 1975).

b. **Growth hormone:** released from the pituitary gland by the Growth Hormone Releasing Hormone GHRH secreted by the hypothalamus usually in response to low blood glucose. It increases blood glucose level by inhibiting cellular uptake of glucose.

c. **Corticosteroids:** they also inhibit cellular uptake of glucose by reducing GLUT-4 transporters. They also enhance the release of glucose from the hepatocytes and stimulate gluconeogenesis and glucagon secretion.

d. **Catecholamines:** include epinephrine and norepinephrine. The former acts via -adrenergic receptors and is from the adrenal medulla while the latter acts via $_2$-adrenergic receptor and is released from the nerve ending. They exhibit opposing effects on insulin release, that is, epinephrine has a stimulatory effect while norepinephrine has an inhibitory effect. The net effect is an increased blood glucose concentration which is achieved via stimulation of glycogenolysis and release of hepatic glucose by epinephrine and indirectly inhibition of insulin release by norepinephrine.

### 2.7.4 Mechanism of Insulin Action

The mechanism of insulin action involves the uptake of glucose by the cells and storage of glucose as glycogen in the liver.

a. **Glucose uptake by cells:** Insulin actions are mediated through the receptors which are embedded in the plasma membrane and composed of two alpha and two beta subunits linked by disulfide bonds. The alpha chains are located extracellularly housing the insulin binding sites while the beta chains penetrate through the plasma membrane.

The insulin receptor is a tyrosine kinase. Functions as an enzyme that transfers phosphate groups from ATP to tyrosine residues in intracellular target proteins. When insulin binds to the alpha subunits, auto-phosphorylation of the beta subunits occurs, thereby activating the receptor which then phosphorylates a number of intracellular proteins which in turn alters their activity, and consequently generates a biological response.

One of the intracellular proteins is Insulin Receptor Substrate 1 (IRS-1) which when activated by phosphorylation trigger a lot of things. IRS-1 serves as a principal agent for activation of other enzymes that mediate insulin's effects.

In the presence of insulin, glucose is taken up by cells by facilitated diffusion through a family of hexose transporters like GLUT4 which is made available in the plasma membrane through insulin action. However, the cells of the brain and liver are independent of insulin in glucose uptake because they are readily permeable to glucose through a non-insulin dependent transporter.

In low insulin concentrations, GLUT4 glucose transporters are present in cytoplasmic vesicles where they are dormant, but once insulin binds to the receptors on such cells these vesicles immediately fuse with the plasma membrane and insertion of the glucose transporters occur, thereby giving the cell an ability to take up glucose effectively. The glucose transporters are recycled back into the cytoplasm when insulin receptors are no longer occupied as a result of decreased blood insulin levels.

b. **Glycogen storage by the liver:** In glycogen synthesis, insulin activates the enzyme hexokinase, which phosphorylates glucose to glucose-6-phosphate, traps it within the cell as well as inhibits glucose-6-phosphatase. Insulin in turn activates other enzymes directly involved in glycogen synthesis, including phosphofructokinase and glycogen synthase. These actions of insulin are targeted at maintaining blood glucose concentrations.

## 2.8 Classification of Diabetes

Diabetes is classified into four broad categories; type 1, type 2, gestational diabetes and other specific types (Shoback, 2011).

a. **Type 1 Diabetes:** it can also be referred to as Insulin-Dependent Diabetes Mellitus (IDDM), early-onset diabetes, juvenile diabetes or childhood onset diabetes. The disease can affect people of any age but it usually occurs in children or young adults (IDF Diabetes Atlas, 2011). Type 1 diabetes is characterized by loss of the insulin-producing beta cells of the islets of Langerhans in the pancreas resulting to insulin deficiency. This type can further be classified as immune-mediated nature, in which beta cell loss is a T-cell-mediated auto-immune attack (Rother, 2007). It is an auto-immune condition in which the insulin producing cells of the pancreas are destroyed and hence the body loses its ability to produce insulin (Bluestone *et al*, 2010). The rate of destruction of the cells is quite variable, being rapid in some individuals and slow in others (Zimmet *et al*, 1994). The rapidly progressive form is commonly observed in children, but also may occur in adults (Humphrey *et al*, 1998).

The slowly progressive form generally occurs in adults and is sometimes referred to as Latent Autoimmune Diabetes in Adults (LADA) (WHO, 1999).

b. **Type 2 Diabetes:** also referred to as Non-Insulin Dependent Diabetes Mellitus (NIDDM) or adult onset diabetes. It is the most common type of diabetes. Type 2 diabetes is characterized by insulin resistance which may be combined with relatively reduced insulin secretion (Shoback, 2011).

Besides family history and genetic factors, risk factors for type 2 diabetes include; obesity, high blood pressure and high cholesterol levels (McCarthy, 2010). Type 2 diabetes occurs when the sensitivity of the peripheral tissues is compromised such that they can no longer respond appropriately to insulin, a condition also known as insulin resistance. Defects in insulin sensitivity and insulin secretion cause glucose to accumulate in the blood (Gastaldelli, 2011). This form of diabetes is frequently undiagnosed for many years because the hyperglycaemia is often not severe enough to provoke noticeable symptoms of diabetes (Harris, 1993; Mooy *et al*, 1995). The disruption of the balance between insulin output and demand aggravates the condition.

c. **Gestational Diabetes:** occurs in about two to five per cent of all pregnancies, it resembles type 2 diabetes as it involves both relative insulin secretion insufficiency and responsiveness. During pregnancy, the pancreatic function might not be sufficient to overcome both the insulin resistance created by the anti-insulin hormone secreted by the placenta (oestrogen, prolactin, human placental lactogen, cortisol and progesterone) (Van der Watt, 2011). It often disappears after delivery. About 20-50 per cent of affected women develop type 2 diabetes later in life. Untreated and poorly controlled gestational diabetes can be fatal both to the foetus and the mother. The baby is at risk of macrosomia (high birth weight), congenital cardiac and central nervous system (CNS) anomalies, skeletal muscle malfunctions, respiratory distress syndrome as a result of inhibition of foetal surfactant production caused by increased foetal insulin, or perinatal death in severe cases resulting from poor placental perfusion caused by vascular impairment.

   Babies born to mothers with gestational diabetes also have a higher risk of obesity and developing type 2 diabetes as adults (IDF Diabetes Atlas, 2011). Individuals at high risk for gestational diabetes include; older women, those with previous history of glucose intolerance, those with history of large for gestational age babies, women from certain high-risk ethnic groups, any pregnant woman who has elevated fasting or casual blood glucose levels (WHO,

1999).

**d.** **Other Specific Types:** other variants of diabetes mellitus are discussed below, and may result from specific genetic conditions, drugs, malnutrition, surgery and other illnesses. These types may account for about 1-5 per cent of all diagnosed cases of diabetes.

Other specific types are currently less common causes of diabetes mellitus but are those in which the underlying defect or disease process can be identified in a relatively specific manner (WHO, 1999). They are as follows;

i. **Genetic defects of beta-cell function:** the defects in the -cell function results to an onset of mild hyperglycaemia at an early age (especially before 25 years). Patients with these forms of diabetes, formerly referred to as maturity-onset diabetes of the young (MODY), have impaired insulin secretion with minimal or no defect in insulin action (Byrne *et al*, 1996; Clement *et al*, 1996).

   The genetic defects of the cells function may also result from abnormalities of the genetic loci in different chromosomes. The most common form is associated with mutations in the following; Chromosome 20, Hepatic Nuclear Transcription Factor HNF4alpha (MODY1), Chromosome 7, Glucokinase (MODY2), Chromosome 12, Hepatic Nuclear Transcription Factor HNF1alpha (MODY3), Chromosome 13, Insulin Promoter Factor IPF-1 (MODY4) and Mitochondrial DNA 3243 mutation.

ii. **Genetic defects in insulin action and processing:** another cause of diabetes as a consequence of genetic abnormalities of insulin action, proinsulin conversion or insulin gene or receptor mutations. The metabolic abnormalities associated with mutations of the insulin receptor may range from hyperinsulinaemia and modest hyperglycaemia to symptomatic diabetes (Tailor, 1992; Kahn *et al*, 1976).

   In the past, this syndrome was termed Type A insulin resistance (Kahn *et al*, 1976). Leprechaunism and Rabson-Mendenhall syndrome are two paediatric syndromes that have mutations in the insulin receptor gene with subsequent alterations in insulin

receptor function and extreme insulin resistance (Tailor, 1992). The former has characteristic facial features while the latter is associated with abnormalities of teeth and nails and pineal gland hyperplasia (WHO, 1999).

iii. **Endocrinopathies:** diseases associated with excess secretion of insulin antagonistic hormones (growth hormone, cortisol, epinephrine, glucagon) can cause diabetes, for example; acromegaly, cushing's syndrome, phaeochromocytoma and glucagonoma. However, when the excess hormone is removed, the hyperglycaemic state resolves. Hyperthyroidism and some tumors (somatostatinoma and aldosteronoma) are also implicated.

iv. **Exocrine pancreatic defects:** direct or indirect injuries to the pancreas can cause diabetes. The factors implicated are as follows; Chronic pancreatitis, Pancreatic neoplasia, Trauma/Pancreatectomy, Cystic fibrosis, Fibrocalculous pancreatopathy, Hemochromatosis.

v. **Infections:** some viruses like the cytomegalovirus, coxsackie virus B and other viruses (like adenoviruses and mumps) have been associated with  cell destruction and hence diabetes induction.

vi. **Drugs or Chemically induced diabetes:** many drugs can impair insulin secretion. These drugs may not, by themselves, cause diabetes but they may precipitate diabetes in persons with insulin resistance (Panditt *et al*, 1993; OøByrne and Feely, 1990). Examples include statins (Sattar *et al*, 2010), glucocorticoids, thyroid hormone, nicotinic acid, vacor (rat poison), pentamidine,  - and  - adrenergic agonists thiazides and so on.

## 2.9 Pathophysiology of Diabetes Mellitus

The pathophysiology of diabetes is based on the interactions of carbohydrate metabolism and insulin. Insulin is the principal hormone regulating glucose uptake from blood into cells of

the liver, muscle and fat (adipose) cells. Therefore, insulin deficiency or insensitivity of its receptors plays a central role in all forms of diabetes.

Moreover, if the production and secretion of insulin are altered by disease, blood glucose dynamics will also change. If insulin production is reduced, glucose entry into cells will be inhibited resulting in hyperglycaemia. The same effect will be seen if insulin is secreted from the pancreas but not used properly by the target cells (Mealey, 2006).

It is worthy of note that glycogenolysis and gluconeogenesis both increase blood glucose levels. Thus, glycaemia is controlled by the complex interactions between the gastrolntestinal tract, the pancreas and the liver. Multiple hormones may affect glycaemia, insulin is the only hormone that lowers blood glucose levels. The counter-regulatory hormones such as glucagon, catecholamines, growth hormone, thyroid hormone and glucocorticoids, all act to increase blood glucose levels in addition to their other effects (Mealey, 2006).

When glucose homeostasis is disrupted, it will no longer be properly absorbed by those body cells that require it. In addition, its storage in the liver and muscles will be disrupted. The net effect is persistent high levels of blood glucose, poor protein synthesis and a host of other metabolic abnormalities or derangements.

## 2.10 Clinical Presentations of Diabetes Mellitus

The symptoms of diabetes vary depending on how high the blood glucose level is elevated.

cell destruction may have started weeks, months or even years before the onset of clinical symptoms. However, symptoms tend to come on quickly and be more severe in type 1 diabetes while in type 2 diabetes , symptoms come on slowly.

These clinical presentations are as follows;

i. **Polyuria (frequent urination):** due to osmotic diuresis secondary to hyperglycaemia.

ii. **Polydypsia (excessive thirst):** due to hyperosmolar state and dehydration.

iii. **Polyphagia (extreme hunger) with weight loss:** weight loss with a normal or increased appetite results from uninhibited gluconeogenesis and high catabolic rate (fat and protein breakdown).

iv. **Fatigue/Weakness:** results from muscle wasting from the catabolic state resulting from insulin deficiency.

v. **Muscle cramps and joint pains:** due to accelerated muscle mass loss, the painful limitation of the shoulder joints may be related to non-enzymatic glycation of proteins.

vi. **Blurred vision:** caused by the hyperosmolar state of the lens and vitreous humor. Glucose and its metabolites cause lens dilatation thereby altering the normal focal length.

vii. **Nocturnal enuresis:** common in young children and secondary to polyuria. It can be a pointer to the onset of diabetes in these children.

viii. **Gastrointestinal disturbances:** like nausea, vomiting, abdominal pain or discomfort, change in bowel movement. In the later stage of diabetes, gastrointestinal disturbances may be due to visceral autonomic neuropathy. Moreover, acute fatty liver may lead to distension of the hepatic capsule resulting in right upper quadrat pain.

ix. **Tingling and numbness in hands or feet:** occurs as a result of peripheral neuropathy occurring in a glove and stocking pattern secondary to accumulation of sorbitol in peripheral sensory nerves due to persistent hyperglycaemia.

## 2.11 Risk Factors for Diabetes

The predisposing factors to the development of diabetes depends on the type of diabetes, this will be listed based on diabetes classification.

**a. Risk factors for type 1 diabetes**

i. Genetic factors.

ii. Presence of Autoantibodies (damaging immune system cells) which results to an autoimmune attack to the pancreas.

iii. Dietary factors: eating the wrong kind of food, like the modern diet which contains processed and junk foods, less nutrient rich foods, high acidic diets and so on. These can cause severe damage to the cells of the pancreas thereby causing the cells to stop or reduce insulin production.

iv. Environmental factors: such as exposure to a viral infection.

**b. Risk factors for type 2 diabetes**

i. Older age: risk of type 2 diabetes increases as one gets older, this may be due to lesser tendency to exercise, loss of muscle mass or weight gain.

ii. Obesity: the more the fatty tissues, the more resistant the cells are to insulin.

iii. Physical inactivity or sedentary lifestyle: physical activity uses up glucose as energy thereby making the cells more sensitive to insulin as well as controlling weight. In other words, risk of type 2 diabetes is increased in the less active persons.

iv. Family history: risk increases when it runs in the family.

v. Race/Ethnicity: blacks, African Americans, Hispanics/Latino Americans, American Indians and Asians and Pacific Islanders have a higher risk of type 2 diabetes.

vi. History of gestational diabetes: women who developed gestational diabetes when pregnant or that gave birth to babies weighing more than four kilograms (nine pounds) are at increased risk of developing type 2 diabetes later in life.

vii. Polycystic ovarian syndrome PCOS: women with PCOS (a condition characterized by irregular menstrual periods, excess hair growth and obesity) are predisposed to type 2 diabetes.

viii. Abnormal cholesterol levels: low levels of high density lipoprotein HDL or õgoodö cholesterol (<35 mg/dl) increases the risk of type 2 diabetes.

ix. High blood pressure: a blood pressure of over 140/90 mmHg predisposes to type 2 diabetes.

x. High levels of triglycerides TG: TG values of above 250mg/dl increases the risk of developing type 2 diabetes.

**c. Risk factors for Gestational diabetes**

i. Obesity: being overweight is associated with a higher risk.

ii. Family or personal history: women with family history of diabetes or had gestational diabetes during previous pregnancies are at a greater risk.

iii. Race: women who are black, Hispanic, American Indian or Asian are more likely to develop gestational diabetes although the reasons are not very clear.

**d. Risk factors for other specific types of diabetes**

Specific genetic syndromes, Surgery, Drugs, Malnutrition, Infections and Other illnesses.

## 2.12 LABORATORY INVESTIGATIONS OF DIABETES MELLITUS

The diagnosis of diabetes mellitus includes some biochemical tests listed below.

**i.** **Fasting Plasma Glucose FPG:** the basic and very important and useful for both diagnosis and follow up. The measurement of fasting glucose gives information on the capability of insulin to control glycogenolysis and gluconeogenesis. When

the production or action of insulin is not sufficient, fasting hyperglycaemia develops. The test is usually performed after an overnight fast, that is, no caloric intake for at least eight hours. FPG ×7.0mmol/l (×126mg/dl) is a criterion for a diabetes diagnosis. However, the test has to be repeated before a confirmation is made. Blood glucose determination is the simplest measurement of carbohydrate homeostasis.

ii. **Random Plasma Glucose RPG:** an alternative test for screening of diabetes. The test is performed at any time of the day irrespective of time of last meal. RPG ×11.1mmol/l (×200mg/dl) is considered diabetes according to the American Diabetes Association ADA.

iii. **Postprandial Plasma Glucose:** is also another alternative clue to diabetes diagnosis. It is performed after meal. Abnormal high values need to be verified by FPG. Normal value <11.1mmol/l (<200mg/dl).

iv. **Oral Glucose Tolerance Test OGTT:** this test examines the efficiency of the body to metabolise glucose, or how quickly it is cleared from blood. It is more sensitive than the previously mentioned. Patient preparation is the major practical concern. Glucose level is measured after an oral glucose load (75g). A fasting period of 8-10 hours is allowed before the test is carried out.

v. **Urine glucose test:** the test detects the presence of glucose in urine which could be suggestive of diabetes mellitus, although a diagnosis must be confirmed by test results showing elevated blood glucose levels.

vi. **Glycated haemoglobin (HbA1c):** this results from the non-enzymatic reaction between glucose and haemoglobin. It is used to determine the glycaemic control over a period of 2-3 months since erythrocytes have an average life span of 120 days. Poor glycaemic control predisposes to development of diabetic

complications. HbA1c is better than fasting glucose for determining risks of CVD and death from any cause (Selvin *et al*, 2010).

Glycosylation of haemoglobin occurs in two steps which results in the formation of covalent bond between glucose molecule and the terminal valine of the chain of the haemoglobin molecule. It is expressed as a per centage of the normal haemoglobin. The values are; HbA1c <6.5 per cent (Good glycaemic control), HbA1c 6.5-8.5 per cent (Moderate glycaemic control) and HbA1c > 8.5 per cent (Poor glycaemic control). A target level of 4.0-6.5 per cent is recommended.

**vii.** **Microalbuminuria test:** a test that detects microquantity of albumin in urine sample. A positive result points to nephropathy or glomerular pathology, a common diabetic complication.

**viii.** **Urine and blood ketone test:** these detect diabetic ketoacidosis which is a specific condition with severe hyperglycaemia, hyperosmolarity and ketone accumulation in patients. The -hydroxybutyrate is specific for diabetic ketoacidosis.

**ix.** **C-Peptide test:** a new test in diabetic medicine which assesses the capacity of the cell. It is also a good diagnostic property for diabetic neuropathy, one of the most common complications of diabetes.

**x.** **Insulin test:** this involves a direct measurement of the hormone which is the major regulator of blood glucose. Apart from the simple insulin levels measurement, the glucose clamp technique is also performed to measure sensitivity and secretion; the hyperinsulinaemic-euglycaemic clamp which assesses in vivo insulin sensitivity and the hyperglycaemic clamp which assesses insulin secretion). The technique assesses the cell function but is difficult and therefore should be carried out by expert.

xi. **Other tests:** these include; Routine blood count and coagulation screen, Arterial blood gases (in emergency cases like ketoacidosis), Serum electrolytes, Urea and Creatinine, Lipid profile, Liver function tests, Thyroid function tests and anti-thyroid antibodies (hyperthyroid children have increased need for insulin and a tendency towards hyperglycaemia (type 1 diabetes). Antithyroid antibody tests indicate the risk of present or potential thyroid disease and Autoantibodies to islet cells (insulin antibodies and antibodies to glutamate decarboxylase (GAD antibodies).

## 2.13 Management of Diabetes Mellitus

The management of diabetes involves maintaining the blood glucose levels close to normal (euglycaemia) without causing hypoglycaemia. This is divided into pharmacological and non-pharmacological approaches.

### 2.13.1 Pharmacological Approach

This involves the use of drugs or medications in achieving euglycaemia in diabetes patients.

a. **Oral medications:** a number of different oral agents are available for treating diabetes, most of these are taken by those with type 2 diabetes (Mealey, 2006).

i. **Sulphonylureas:** drugs for type 2 diabetes treatment (examples; glibenclamide, chlopropamide). They stimulate pancreatic insulin secretion by closing the ATP-sensitive potassium channel on the cell membrane thereby depolarizing it so that insulin is released. The increased quantity of secreted insulin helps counteract the quantitative decrease in tissue sensitivity to insulin, allowing greater glucose entry into target cells and thereby lowering blood glucose levels (Mealey, 2006).

The major side effect in patients taking these drugs is hypoglycaemia. But in order to avert this, adequate food intake must be ensured.

ii. **Biguanides:** they enhance insulin sensitivity and lowers blood glucose by increasing utilization of glucose by skeletal muscle and decreasing hepatic gluconeogenesis and glycogenolysis. Metformin is the most widely used and frequently prescribed to overweight patients as it also promotes weight loss (Van der Watt, 2011).

iii. **Thiazolidinediones:** increase insulin sensitivity by increasing utilization of glucose and decreasing hepatic gluconeogenesis, thus decreasing blood glucose levels. Examples are Rosiglitazone, Pioglitazone and Troglitazone.

**b. Insulin**: exogenous insulin is used in the treatment of type 1 diabetes as well as type 2 diabetes when oral hypoglycaemic agents become ineffective. The most common complication of insulin therapy is hypoglycaemia which is a life-threatening emergency. The hypoglycaemia occurring in patients using insulin is more common than those taking oral agents like sulphonylureas.

Intensified treatment regimens for diabetes increase the risk of hypoglycaemia. Thus, the long term benefit of reduced diabetic complications seen in intensive treatment must be weighed against the risk of symptomatic risk of hypoglycaemia (Mealey, 2006) such as seizures, unconsciousness and brain damage and so on.

### 2.13.2 Non-Pharmacological Approach

This involves changes in lifestyle as follows;

**a. Diet:** caloric restriction is of utmost importance in management of diabetes patients. Intake of high caloric diets like simple sugars and saturated fats should be minimized if not avoided while diets rich in vitamins and minerals should be encouraged. Timing, size, frequency or composition of meals must also be considered in order to avoid hypoglycaemia or postprandial hyperglycaemia.

**b. Physical activity or Exercise:** exercise improves insulin sensitivity thereby keeping short and long term blood glucose levels within acceptable limits. However, very stressful

exercise is unacceptable because this may trigger the release of the ÷stressø hormone cortisol.

c. **Weight reduction:** excess weight or obesity which is very common in type 2 diabetes contributes to insulin resistance. Therefore, weight reduction improves the sensitivity of target cells to insulin.

## 2.14 Diabetic Complications

Diabetes mellitus is associated with an increased risk of developing certain health problems which may be disabling or even life threatening. Persistent or poorly controlled hyperglycaemia can lead to serious life complicating diseases affecting the kidney, nerves, eyes, heart and blood vessels.

### 2.14.1 Classification of Diabetic Complications

Complications of diabetes can be classified as acute (short term) or chronic (long term) complications.

### 2.14.2 Acute Complications

These are life threatening conditions resulting from diabetes, and occur over a short period of time. It can also be referred to as Diabetic Emergencies. These are discussed below.

i. **Diabetic Ketoacidosis (DKA):** this is an acute complication of diabetes which is usually very fatal if not promptly diagnosed and treated. It occurs in both types 1 and 2 diabetes mellitus. This condition may occur as a result of insulin deficiency (relative or absolute) in combination with excess of insulin antagonistic hormones (glucagon, catecholamines, cortisol and growth hormone).

   The ratio of insulin to glucagon is decreased, which triggers gluconeogenesis, glycogenolysis and ketone bodies formation. It presents with the following; nausea, vomiting, thirst, shortness of breath, polyuria, cerebral edema, coma, dehydration, hyperosmolarity, lethargy, ketonuria and ketonaemia.

ii. **Hyperglycaemic Hyperosmolar State (HHS):** it can also be referred to as Non-ketotic Hyperglycaemic State. It is an acute complication of diabetes mainly seen in type 2 diabetes patients. This can be caused by relative insulin deficiency and inadequate fluid intake. Deficiency of insulin promotes hepatic glucose production (through gluconeogensis and glycogenolysis). However, blood and urine ketones are absent.

Hyperglycaemia induces osmotic diuresis that leads to intravascular volume depletion which is worsened by inadequate fluid replacement. Severe dehydration causes hyperviscocity of blood which leads to decreased tissue perfusion with lactic acidosis, cerebral thrombosis and renal shutdown. HHS presents with; polyuria, weight loss, lethargy, severe dehydration, hyperosmolarity, mental confusion.

### 2.14.3 Chronic Complications

These are long term complications resulting from persistent, uncontrolled or poorly controlled hyperglycaemia. They are divided into three; microvascular, macrovascular and others.

### 2.14.4 Microvascular Complications

These affect smaller blood vessels and nerves and are discussed below.

a. **Diabetic Retinopathy:** diseases of the eye which can hinder sight or cause blindness. Chronic hyperglycaemia can block and damage the network of blood vessels that supply the retina as a result of precipitating hyperglycaemia. These small blood vessels include retinal precapillaries, arterioles, capillaries and venules. Early pathological features include thickening of the basement membrane which may impair oxygen diffusion, loss of pericytes and development of microaneurysms. The major eye problems related to diabetes are glaucoma, cataract and retinopathy (Dwi, 2012).

The accumulation of sorbitol in lenses has also been implicated in cataract formation in diabetes patients. In addition to hyperglycaemia, high cholesterol and high blood pressure also contribute to retinopathy leading to permanent vision loss. As a result of this occlusion, the short or cut in blood supply to the retina leads to retinal ischaemia, this triggers the release of cytokines which promotes neovascularisation (development of new blood vessels). This is classified into proliferative and non-proliferative.

i. **Proliferative diabetic retinopathy:** this type involves the appearance of new blood vessels in response to retinal ischaemia which appear near the optic nerve and easily ruptures causing vitreous haemorrhage, fibrosis and finally retinal detachment.

ii. **Non-proliferative Diabetic Retinopathy:** this is characterised by intraretinal microvascular abnormalities, microaneurysms and haemorrhages.

**b. Diabetic Nephropathy:** the kidneys contain millions of tiny blood vessels (tuft of capillaries) in the glomeruli which filter the waste from blood. Diabetes can damage this filtering system leading to kidney failure or End Stage Renal Disease (ESRD).

Like other microvascular complications, chronic hyperglycaemia is the fundamental cause of diabetic nephropathy. Excretion of micro quantities of albumin (about 30-300 milligram albumin per day) is an early indicator. Persistent albuminuria is accompanied by a gradual decline in glomerular filtration rate GFR.

c. **Diabetic Neuropathy:** nerve damage caused by hyperglycaemia resulting from the formation of sorbitol caused by the accumulation of glucose in nerve cells. Sorbitol interferes with uptake of inositol which is required for nerve signal transduction. It can affect nerves in different parts of the body.

Conditions arising from diabetic neuropathy include problems with digestion, passing urine, impotence. Peripheral neuropathy involves the nerve damage in the extremities, especially the feet which can cause pain, tingling and loss of sensation (numbness). This

is a very serious condition because injuries in these parts of the body may not be noticed and treated early, and therefore can lead to toe, foot or even leg amputation. Poor circulation can also cause cramps in the calves.

### 2.14.5 Macrovascular Complications

These are life-threatening conditions resulting from damage to the heart and larger blood vessels. Hyperglycaemia, increased cholesterol and triglycerides, high blood pressure and other predisposing factors contribute to the increased risk of cardiovascular disease. The various cardiovascular problems resulting from diabetes are listed below.

a. **Coronary Heart Disease:** those affecting the heart and coronary circulation, thickening of coronary artery walls or clot formation in these arteries reduce blood flow to the heart, causing strain on the heart which eventually leads to angina (chest pain), myocardial infarction (heart attack) or sudden death. Sustained hyperglycaemia such as in the case of diabetes could lead to hypertension and heart valve defects. These conditions eventually manifest as cardiac hypertrophy which is the thickening of the myocardial wall, and hence reduction in the ventricular chambers (Dwi, 2012).

b. **Cerebrovascular Diseases:** those affecting the brain and cerebral circulation. Diminished or interrupted blood supply to the brain as a result of cerebral thrombosis or haemorrhage will definitely result to a stroke, immediate loss of function of part of the brain resulting in death (infarction) of an area within the brain (IDF, 2011).

c. **Peripheral Vascular Disease:** those affecting lower limbs and feet. This results from the narrowing of blood vessels supplying the leg and arm muscles. The diminished blood supply to these areas can cause gangrene (death of tissue leading to amputation) and pain in the calves while walking.

### 2.14.6 Other Complications

These include other disease conditions resulting from diabetes.

Gastroparesis, Infections, Gum or Periodontal diseases, Poor mental health, Delayed wound healing, Sexual problems and Liver problems.

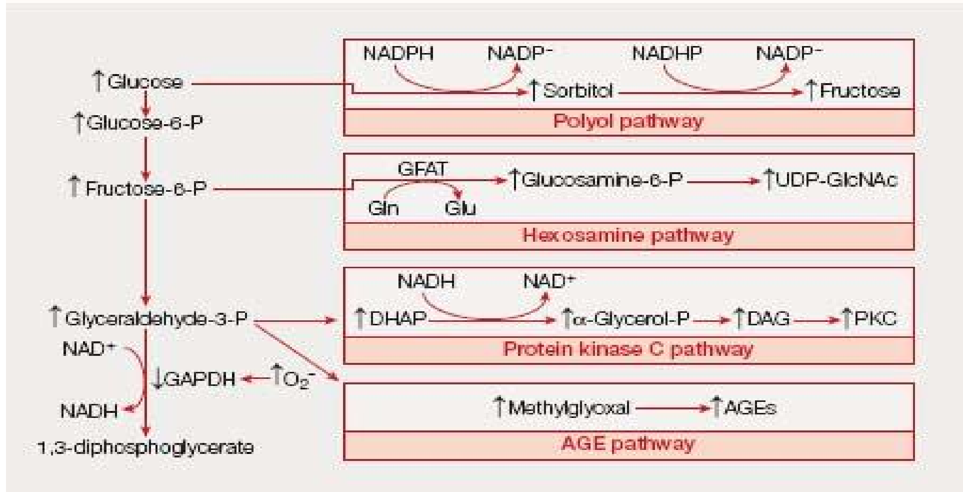
### 2.14.7 Pathogenesis of Diabetic Complications

Hyperglycaemia dramatically alters the function of multiple cell types and their extracellular matrix and also results in structural and functional changes in the affected tissues (Mealey, 2006). Recent evidence suggests that glucose overload may damage cells through oxidative stress, and this hyperglycaemia-induced oxidative stress may account for the pathogenesis of all diabetic complications. These damages occur in a particular subset of cells which include capillary endothelial cells in the retina, mesangial cells in the renal glomerulus, and neurons and Schwann cells in peripheral nerves (Brownlee, 2001).

Many biochemical pathways associated with hyperglycaemia increase the production of free radicals leading to oxidative stress, including glucose auto-oxidation, the polyol pathway, prostanoids synthesis, protein glycation and the protein kinase C (PKC) pathway (Giugliano *et al,* 1996). It has also been stated that the overproduction of superoxide ion is the principal activator of all other pathways.

The four key biochemical hyperglycaemia-induced changes are discussed below. Nishikawa *et al* (2000) proposed the existence of a unifying mechanism that integrates the four biochemical pathways.

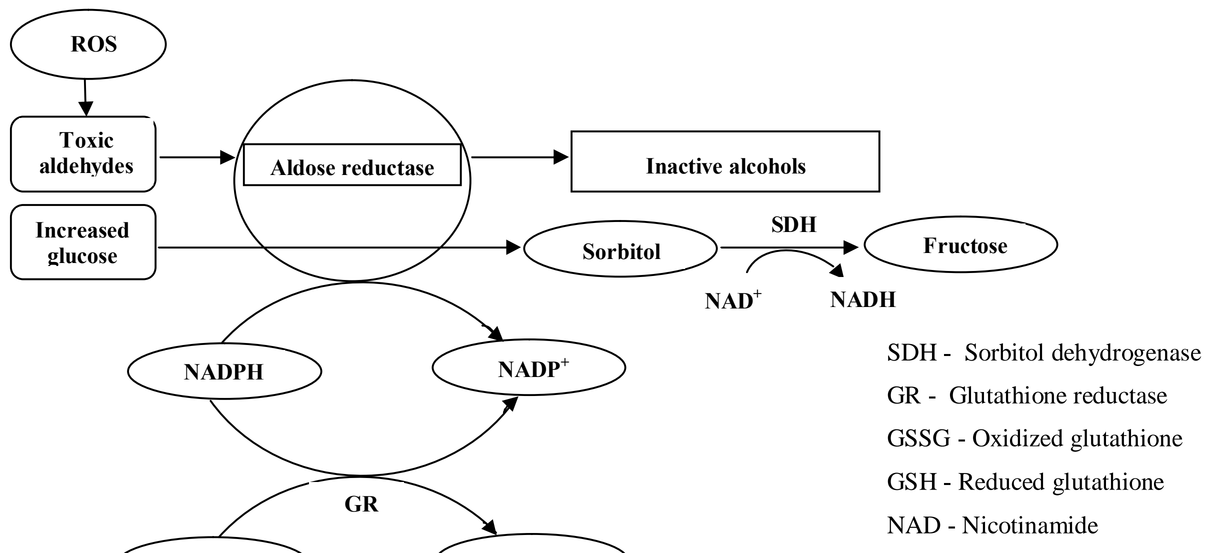
**Figure 2.2: Unifying mechanism integrating the four pathways**




**a. The Polyol Pathway:** this focuses on the enzyme aldose reductase which normally reduces toxic aldehydes in the cell to inactive alcohols. Aldose reductase reduces glucose to sorbitol, which is later oxidized to fructose when glucose concentration in the cell rises. This leads to the formation of cataracts in lens and peripheral neuropathy.

In the reduction of glucose to sorbitol, aldose reductase makes use of the co-factor Nicotinamide Adenine Dinucleotide Phosphate (NADPH) which is necessary for generating reduced glutathione GSH (an important intracellular antioxidant). Hence, a reduction in the levels of NADPH and GSH increases susceptibility to intracellular oxidative stress.

**Figure 2.3: Hyperglycaemia increases flux through the Polyol pathway**



In the aldose reductase osmotic hypothesis, accumulation of polyols initiates lenticular osmotic changes (Maritim *et al,* 2003).

b. **The Hexosamine pathway:** this involves the shunting of excess glucose through the hexosamine pathway. When glucose concentration inside the cell is high, most of the glucose is metabolized through the glycolytic pathway, from glucose-6-phosphate, fructose-6-phosphate and so on. Some of the fructose-6-phosphate is converted to glucosamine-6-phosphate catalyzed by GFAT and finally to UDP-GlcNAc. The N-acetyl glucosamine then links to serine and threonine residues of transcription factors just like the more familiar process of phosphorylation, and over modification by this glucosamine often results in pathologic changes in gene expression (Kolm-Litty *et al,* 1998; Sayeski and Kudlow, 1996; Wells and Hart; 2003). Increased modification of the transcription factor results in increased expression of transforming growth factor- 1 and plasminogen activator inhibitor-1(PAI-1), both of which are bad for diabetic blood vessels. Increased glucose can also increase transcription of fibronectin, 1-laminin and type IV collagen.

c. **Activation of Protein Kinase C (PKC) pathway:** PKC activation effects range from vascular occlusion to pro-inflammatory gene expression. It also decreases some positive factors necessary for normal body functions and vice versa. For example, the vasodilator, endothelial nitric oxide synthase (eNOS) is decreased while the vasoconstrictor endothelin-1 is increased. In this glucose-mediated activation of PKC, glucose is converted to DAG, a PKC activator, resulting in basement membrane and matrix production, altered enzyme activity, cell proliferation and increased blood flow.

d. **The AGE pathway:** is also known as non-enzymatic glycosylation or Maillard reaction. Increased formation or production of advanced glycation end products or precursors damage cells through three mechanisms.

Modification of intracellular proteins including those involved in gene transcription.

Modification of nearby extracellular matrix molecules when they diffuse out of the cell which changes the signal between the matrix and the cell resulting in cellular dysfunction.

Modification of circulating proteins can then bind to AGE receptors and activate them, thereby causing the production of inflammatory cytokines and growth factors, which in turn cause vascular pathology (Smedsrod *et al,* 1997; Vlassara *et al,* 1995).

In addition, these AGEs, via their receptors (RAGEs), inactivate enzymes and alter their structures and functions (Mc Carthy *et al,* 2001), promote free radical formation (Baynes and Thorpe, 1999), and quench and block anti-proliferative effects of nitric oxide (Vlassara, 1997; Wautier *et al,* 1994).

By increasing intracellular oxidative stress, AGEs activate the transcription factor nuclear factor kB (NF-kB), thus promoting up-regulation of various NF-kB controlled target genes (Mohammed *et al,* 1999). NF-kB enhances production of nitric oxide (NO), which is believed to be a mediator of islet beta cell damage (Maritim *et al,* 2003). Reducing sugars like glucose binds covalently to the amino groups (example lysine) by means of ketoamine bond. This can also happen in DNA, proteins and lipids resulting to the formation of Amadori products (like glycosylated hemoglobin HbA1c) which impair enzyme activities, formation of glycated low density lipoproteins LDL compounds not recognised by receptors, and therefore are trapped in extracellular matrices and arteries.

### 2.14.8 Prevention of Diabetic Complications

The occurrence of diabetic complications in diabetes patients can be reduced or minimized through the following ways according to the National Diabetes Facts Sheet, 2003.

a. **Blood glucose control:** an improved or good glycaemic control is necessary for the prevention of complications of diabetes. Generally, for every 1% reduction in results of HbA1c blood tests (from 8 to 7 per cent) reduces the risk of developing microvascular diabetic complications (eye, kidney, and nerve disease) by 40 per cent.

b. **Blood pressure control:** control of blood pressure can reduce the risk of cardiovascular disease by approximately 33 to 50 per cent, and microvascular disease (eye, kidney, and nerve disease) by approximately 33 per cent. Generally, for every 10 millimeters of mercury (mmHg) reduction in systolic blood pressure, the risk of any diabetic complication reduces by 12 per cent.

c. **Control of blood lipids:** improved control of cholesterol or blood lipids (example; HDL, LDL, and triglycerides) can reduce cardiovascular complications by 20 to 50 per cent.

d. **Preventive care for the eyes:** detecting and treating diabetic eye disease with laser therapy can reduce the risk of blindness by 50 to 60 per cent.

e. **Preventive care for the kidneys:** detecting and treating early diabetic kidney disease by lowering blood pressure can reduce the decline in kidney function by 30 to 70 per cent. Treatment with angiotensin converting enzyme ACE inhibitors and angiotensin receptor blockers (ARBs) are more effective in reducing the decline in kidney function than other blood pressure lowering drugs.

f. **Preventive care for the feet:** comprehensive foot care programs can reduce amputation rates by 45 to 85 per cent.

#### 2.14.9 Oxidative Stress in Diabetes

Oxidative stress is thought to contribute to the development of a wide range of diseases including the pathologies caused by diabetes (Davis *et al,* 2005). For the purpose of this write up, oxidative stress will be discussed in relation to diabetes and its complications.

Hyperglycaemia alters reactive oxygen species (ROS) production, particularly in the mitochondria, leading to increased intracellular ROS and activated stress-sensitive pathways such as nuclear factor kB (NF-kB), p38 mitogen-activated protein kinase (MAPK), and the c-Jun $NH_2$-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathways (Johansen *et al,* 2005). Subsequently, PKC activity, advanced glycation end-products (AGEs) and sorbitol

levels increase and this can lead to more ROS generation in a positive regulatory feedback loop to chronically stimulate stress-sensitive pathways (Sih MinTan *et al,* 2012).

Under physiological conditions, reactive oxygen and reactive nitrogen species (RNS) are produced and maintained at steady-state levels within a cell (Lushchak, 2011). On the other hand, oxidative stress arises when an imbalance occurs between the production of ROS/RNS and the antioxidant defences that neutralise them, shifting the balance in favour of enhanced ROS levels. The consequence of this shift is cellular damage to biologically important molecules and organelles (Sies, 1997). Elevations in ROS/RNS levels are mainly caused by an imbalance between the activity of endogenous pro-oxidant enzymes, such as NADPH oxidase, xanthine oxidase or the mitochondrial respiratory chain, and the antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), heme oxygenase (HO), thioredoxin (Trx) peroxidase/peroxiredoxin, catalase and paraoxonase (Forstermann, 2008).

ROS include the superoxide anion ($^{\cdot}O_2^-$) and the hydroxyl radical ($^{\cdot}OH$), as well as non-radical species such as hydrogen peroxide ($H_2O_2$).

RNS include the free radicals nitric oxide (NO) and non-radical species such as peroxynitrite ($ONOO^-$) and nitrogen dioxide ($NO_2$) (Johansen *et al,* 2005). In a hyperglycaemic milieu, $^{\cdot}O_2^-$ increases through the enhanced activity of enzymatic sources, including NADPH oxidase and xanthine oxidase, and non-enzymatic sources such as the mitochondrial respiratory chain, glucose auto oxidation, AGE formation and activation of the polyol pathway.

In addition, antioxidant defences are known to decrease in a hyperglycaemic milieu, shifting the balance away from steady-state levels of ROS towards an environment of oxidative stress.

## 2.15 Antioxidants Defence in Diabetic Complications

Evidences suggest that glucose alters antioxidant defences in endothelial cells (Ceriello *et al,* 1996) and in patients with diabetic complications such as diabetic nephropathy (Ceriello *et al,*

*2000;* Hodgkinson *et al,* 2003). The concentration of the antioxidant glutathione (GSH) is found to decrease in a range of organs including the liver, kidney, pancreas, plasma and red blood cells of chemically induced diabetic animals (Maritim *et al,* 2003b).

Given that reduced GSH functions as a direct free-radical scavenger and a co-substrate for glutathione peroxidase (GPx) activity, as well as a co-factor for many enzymes, reductions in this antioxidant induced hyperglycaemic environment is likely to impact on the progression of diabetic complications (Sih Min Tan *et al,* 2012).

These findings therefore suggest that increased ROS in diabetes is not only the result of their increased production but also a consequence of impaired antioxidant defenses.

**Figure 2.4: Antioxidant defense in diabetic complications**

### 2.15.1 Glutathione Peroxidase (Gpx)

It is a seleno-cysteine-containing antioxidant enzyme which attenuates oxidative stress by reducing hydrogen and lipid peroxides to water and their corresponding alcohol using GSH. Pre-clinical and clinical evidences are now mounting in support of the important role of GPx in the protection against diseases such as atherosclerosis both in diabetic and non-diabetic setting. Thus, the major role of GPx in the protection against pathogenesis may reside in the fact that it is the only antioxidant enzyme that metabolizes three major ROS; hydrogen peroxide ($H_2O_2$), lipid peroxide (LOOH) and peroxynitrite ($ONOO^-$) (Sih Min Tan *et al,* 2012). Several isoforms of GPx have been identified and they are each encoded by separate genes, which vary in cellular location, substrate specificity and tissue specific functions (Brigelius-Flohé, 1999).

- **a. GPx1:** this is also known as cellular GPx, and first identified as an erythrocyte enzyme which protects the haemoglobin from oxidative injury. Its ubiquitous expression in almost all tissues together with its abundant expression in organs such as kidney and liver have meant that this isoform is one of the most well characterized of the GPx family (Lei, 2001).
- **b. GPx2:** it is mostly found in the gastrointestinal tract, and protects the intestinal epithelium from oxidative stress.
- **c. GPx3:** it is secreted by the kidney and is the main source of plasma GPx, it is also expressed in the other tissues, for example in the heart (Reeves and Hoffman, 2009).
- **d. GPx4:** this reduces phospholipid hydroperoxides (Conrad *et al*, 2007; Thomas *et al*, 1990) and is thought to play a protective role in oxidative stress-induced apoptosis, possibly through the mitochondrial death pathway (Nomura *et al*, 1999; Seiler *et al*, 2008).

The decrease in GPx activity may contribute to the progression of diabetic complications due to the build-up of ROS such as $H_2O_2$ and $ONOO^-$, leading to lipid peroxidation and oxidative injury (Sih Min Tan *et al*, 2012).

### 2.15.2 Superoxide Dismutase (SOD)

SOD is an antioxidant enzyme that catalyzes the conversion of superoxide anion ($^{\cdot}O_2^-$) to hydrogen peroxide and oxygen thus, removing $^{\cdot}O_2^-$. There are three isoforms of SOD in humans with their distinct cellular localization. These are SOD1, also known as Cu/Zn-SOD, located in the cytosol, SOD2, also known as Mn-SOD located in the mitochondria and SOD3 located extracellularly.

Diabetes is associated with a decrease in SOD activity in most animal studies (Brocca *et al*, 2008; Fujita *et al*, 2009; Fukuda *et al*, 2010). Studies have also shown that targeted removal of $^{\cdot}O_2^-$ leads to improved outcomes in diabetic complications like diabetic nephropathy and diabetic retinopathy.

### 2.15.3 Catalase

Catalase is present mainly in the peroxisomes of mammalian cells as a tetrameric enzyme of four identically arranged subunits; each containing a heme group and NADPH at its active centre (MatÉs *et al,* 1999). It catalyzes the conversion of hydrogen peroxide to water and oxygen.

### 2.15.4 Thioredoxin (Trx) System

The mammalian Trx system is ubiquitously expressed and consists of Trx, Trx reductase and NADPH. The antioxidant properties of Trx is exerted mostly through the antioxidant enzyme, Trx peroxidase (also known as Peroxiredoxin), uses sulfhydryl (SH) groups as reducing equivalents. Trx reduces oxidized peroxiredoxin, which then scavenges hydrogen peroxide to produce water (Kang *et al,* 1998), thus attenuating oxidative stress in cells. The role of the Trx system in diabetic complications has gained considerable interest since thioredoxin-interacting protein (Txnip), which is an inhibitor of Trx activity was discovered to be a highly upregulated hyperglycaemia-induced gene in both human and animal studies (Kobayashi *et al,* 2003; Qi *et al,* 2007; Shalev *et al,* 2002). Txnip directly binds to the catalytic active site of Trx, thus inhibiting the reducing activity of Trx (Nishiyama *et al,* 1999).

## 2.16 The Enzyme Gamma Glutamyl Transferase (GGT)

It is also known as gamma glutamyl transpeptidase GGTP, an enzyme that catalyzes the transfer of gamma glutamyl functional groups. The enzyme is found in cell membrane of many tissues like liver, heart, brain, bile ducts, pancreas, spleen, kidneys and seminal vesicles.

### 2.16.1 Functions of GGT

GGT is involved in the transfer of amino acids across cell membrane. It is also involved in glutathione metabolism by transferring the glutamyl moiety to a variety of acceptor molecules including water, certain L-amino acids and peptides, leaving the cysteine product to preserve intracellular homeostasis of oxidative stress (Yokoyama, 2007).

### 2.16.2 Clinical Applications of GGT

GGT has several uses as a diagnostic marker in medicine. It has long been used as a marker of liver dysfunction. Elevated serum GGT activity can be found in diseases of the liver, kidney, biliary system and pancreas.

GGT has also been used as a marker of alcohol intake and a monitor of return to drinking by those who previously quitted drinking.

The last few years have seen improvements in these areas and advances in understanding of its physiological role in counteracting oxidative stress by breaking down extracellular glutathione and making its component amino acids available to the cells (Whitfield, 2001).

Expression of GGT is enhanced by oxidative stress, and it could be released by several conditions including cellular stress. Serum GGT concentrations have recently been associated with cardiovascular disease risk factors or components of metabolic syndrome.

However, conditions that increase serum GGT, such as obstructive liver disease, high alcohol consumption, and use of enzyme-inducing drugs, lead to increased free radical production and the threat of glutathione depletion (Whitfield, 2001). Glutathione plays an important role

in protecting cells against oxidants that are produced during normal metabolism. If oxidative stress increases, then the requirement for reduced glutathione also increases, but when this is not available, the effects of oxidative stress become greater and devastating.

There is clear evidence that cellular GGT level is closely related to oxidative stress indicators in vivo, either as an antioxidant or a prooxidant, depending on circumstances (Hsueh and Quinones, 2003).

Elevated GGT could reflect subclinical inflammation, which would represent the underlying mechanism. In addition, certain mechanisms related to oxidative stress might play a role because cellular GGT has a central role in glutathione homeostasis by initiating the breakdown of extracellular glutathione, a critical antioxidant defense for the cell (Burkitt *et al,* 1993).

Increases in serum GGT activity may be a response to oxidative stress, making increased transport of glutathione into cells (Green *et al,* 2003).

# CHAPTER THREE

# MATERIALS AND METHOD

## 3.1 Experimental Animals

40 albino wistar rats of both sexes weighing between 160g and 210g were used for this study. They were obtained and housed at room temperature with 12-hour light and dark cycles in the animal house of College of Medicine, University of Nigeria, Enugu Campus.

The animals were maintained on a standard poultry diet (Vital Feeds, Jos) and water *ad libitum*, and were allowed an acclimatization for a period of two weeks.

They were handled according to the institutional and international guidelines for the care and use of laboratory animals.

## 3.2 Experimental Design

The animals were randomly assigned into two groups A and B consisting of twenty rats each. Group A was the control (non-diabetic) group, while Group B was the experimentally-induced diabetic group.

## 3.3 Induction of Experimental Diabetes

After an overnight fast, experimental diabetes was induced in group B animals by a single intraperitoneal dose of alloxan monohydrate at 150mg/kg body weight dissolved in freshly prepared normal saline while those in group A were given normal saline intraperitoneally after an overnight fast. Development of diabetes was confirmed one week post-alloxan administration in Group B after an overnight fast by measuring the fasting plasma glucose (FPG) levels using a commercial glucose kit (Randox). Only rats with FPG above 11mmol/l were included in this study.

On the 3rd and 5th weeks respectively, 10 rats from each group were fasted overnight, euthanized, and blood samples collected through cardiac puncture for the estimation of biochemical parameters and organs excised for histological studies.

The body weights of the rats were measured using a spring balance before and at the end of the experiment.

### 3.4 Biochemical Assays

Blood samples were collected in three different test tubes; fluoride oxalate tube for the estimation of plasma glucose, plain tubes (without anticoagulants) to separate serum for estimation of some biochemical parameters and EDTA tubes for glycated haemoglobin (HbA1c) estimation. Blood samples in fluoride oxalate and plain tubes were centrifuged in Techmel and Techmel 800D centrifuge USA at 3000g for 5 and 10 minutes respectively.

The supernatant plasma was used for FPG assay while the serum was used for urea, creatinine, ALT, AST, lipid profile, GGT and TAC assay.

#### 3.4.1 FPG Assay

This was done using the glucose oxidase method as described by Trinder, 1962.

**Principle:** glucose is oxidized by glucose oxidase to gluconic acid and hydrogen peroxide. The latter is then oxidatively coupled with a chromogen to produce a coloured compound which is measured colourimetrically.

**Procedure:** 1000 l of the working reagent (Randox kit) was added to 10 l of sample and standard, this was mixed and incubated for 20 minutes at 25°C (room temperature) and absorbance of sample and standard were measured against the reagent blank at 520nm.

### 3.4.2 Urea Assay

The diacetylmonoxime (DAM) method was used.

**Principle:** DAM is hydrolyzed to diacetyl and monoxime in a hot acid medium, diacetyl condenses with urea to form a pink coloured complex whose intensity is proportional to the urea concentration.

**Procedure:** 2000 l each of the mixed acid and colour were added to 10 l of sample and standard mixed and incubated at 100$^{o}$C for 10 minutes. Absorbances were read at 540nm after cooling.

### 3.4.3 Creatinine Assay

This was according to Jaffeøs method as modified by Fabing and Ertingshaushen, 1971.

**Principle:** creatinine reacts with picric acid in an alkaline medium to form a coloured complex of creatine picrate.

**Procedure:** 1500 l of distilled water was added to 500 l of both serum and standard while 2000 l of distilled water was added to the blank. 1000 l each of $H_2SO_4$ and sodium tungstate were added to sample, standard and blank, incubated at room temperature and then centrifuged for 10 minutes. 500 l each of NaOH and picric acid were added to 1500 l of the supernatant, incubated at room temperature for 15 minutes and read at 490nm.

### 3.4.4 Alanine and Aspartate Transaminases (ALT & AST)

This was estimated according to the method described by Rietman and Frankel, 1954.

**Principle:** transaminases (metabolic enzymes) which catalyze transamination reactions in the body lead to the production of keto-acids (pyruvate and oxaloacetate with ALT and AST respectively) which couples with 2,4-Dinitrophenylhydazine to form hydrazones whose colour intensity is proportional to the amount of keto-acids formed which in turn depends on the enzyme activity in the sample.

**Procedure:** 500 l each of ALT and AST substrates were incubated for 5 minutes at 37$^{o}$C (for Test and Blank).

For Test: 100 l and 200 l of samples were added to the ALT and AST substrates respectively, mixed and incubated for 30 minutes at 37$^{o}$C. 500 l of 2,4-DNPH was added to both Test and Blank.

For Blank: samples were added as in Test above to the substrate/DNPH mixture and incubated for 20 minutes at room temperature. 5000 l of NaOH was added to both Test and Blank, and absorbance read at 540nm.

### 3.4.5 Total Cholesterol (TC) Assay

This was estimated as described by Richmond, 1973 and Roeschlau *et al*, 1974.

**Principle:** cholesterol is determined after enzymatic hydrolysis and oxidation, the indicator quinoneimine is formed from $H_2O_2$ and 4-aminoantipyrine in the presence of phenol and peroxidase.

**Procedure:** 1000 l of cholesterol reagent (Randox kit) was added to 10 l each of the sample and standard, mixed and incubated for 10 minutes at room temperature. The absorbances were measured against the reagent blank at 540nm.

### 3.4.6 High Density Lipoprotein Cholesterol (HDL-C) Assay

This was measured using the method of Wacnic and Alber, 1978.

**Principle:** low density lipoproteins (LDL and VLDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. After centrifugation, the cholesterol concentration in the HDL fraction, which remains in the supernatant, is determined.

**Procedure:** 500 l of diluted precipitant and 200 l of sample and standard were mixed and incubated for 10 minutes at room temperature, then centrifuged for 10 minutes. 1000 l of cholesterol reagent (Randox kit) was added to 10 l each of the supernatant and standard, mixed and incubated for 10 minutes at room temperature, then read colorimetrically at 540nm.

### 3.4.7 Triglyceride (TG) Assay

This was estimated according to the method described by Tietz, 1990.

**Principle:** TG measurements are determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from $H_2O_2$, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.

**Procedure:** 1000 l of the TG reagent (Randox kit) was added to 10 l each of sample and standard, incubated for 10 minutes at room temperature, and absorbance read at 540nm.

### 3.4.8 Low Density Lipoprotein Cholesterol (LDL-C) Assay

This was calculated in mmol/l using the Friedewaldøs equation (1972) as stated below:

$$\text{LDL-C} = \text{TC} - \left(\text{HDL-C} + \frac{\text{TG}}{2.2}\right)$$

### 3.4.9 Very Low Density Lipoprotein Cholesterol (VLDL-C) Assay

This was also calculated in mmol/l using the Friedewaldøs equation (1972) as stated below:

$$\text{VLDL-C} = \frac{\text{TG}}{2.2}$$

### 3.4.10 Gamma-Glutamyl Transferase (GGT) Assay

This was measured as described by Gendler *et al,* 1984 and Persijn *et al,* 1976.

**Principle:** GGT catalyzes the transfer of gamma- glutamyl group from gamma-glutamyl-p-nitroaniline to an acceptor glycylglycine. The coloured complex formed is proportional to the catalytic concentration of GGT in the sample.

**Procedure**: Samples were analyzed using Mindray BS-120 auto analyser and colour intensity measured photometrically.

### 3.4.11 Total Antioxidant Concentration (TAC) Assay

The TAC of serum was estimated as described by Koracevic *et al,* 2001.

**Principle:** The assay measures the capacity of serum to inhibit the production of thiobarbituric acid reactive substance (TBARS) from sodium benzoate under the influence of

the oxygen radicals derived from the Fentonøs reaction. Antioxidants from the added sample causes suppression of the production of TBARS, and the inhibition of colour development is defined as the antioxidant activity.

**Procedure:** six test tubes labeled sample (**$A_1$**), sample blank (**$A_0$**), negative control (**$K_1$** and **$K_0$**) and standard (**$UA_1$** and **$UA_0$**) were used, in which each sample was analyzed in six (6) runs with negative control for each series of analysis prepared in duplicate containing the same reagents as **$A_1$** and **$A_0$** except for serum.

10  l of serum was added to $A_1$ and $A_0$, 10  l of uric acid was added to $UA_1$ and $UA_0$ after which 490  l of buffer, 50  l of sodium benzoate, 200  l of iron-EDTA and 200  l of hydrogen peroxide were added to $A_1$, $A_0$, $K_1$, $K_0$, $UA_1$ and $UA_0$. 1000  l of acetic acid was then added to $A_0$, $K_0$ and $UA_0$. The solutions were mixed and incubated at 37$^{o}$C before adding 1000  l of acetic acid to $A_1$, $K_1$ and $UA_1$, and 1000  l of thiobarbituric acid to $A_1$, $A_0$, $K_1$, $K_0$, $UA_1$ and $UA_0$.

### 3.4.12 Glycated Haemoglobin (HbA1c) Assay

This was measured in whole blood using the micro-column chromatography method as described by Bissé and Abragam, 1985.

**Principle:** after preparing the haemolysate where the labile fraction is eliminated, haemoglobins are retained by a cationic exchange resin. HbA1c eluted after washing away the HbA1a+b fractions is quantified by direct photometric reading.

**Procedure:** the columns and reagents were brought to room temperature. 50  l blood and 200  l reagent 1 were mixed (haemolysate) and incubated for 15 minutes at room temperature. 50  l of haemolysate and 200  l reagent 2 were added to each column and left to drain to waste. The columns were then placed over new test tubes and 4ml reagent 3 was added, and the absorbance of the eluate ($A_{HbA1c}$) collected read at 415nm. 12ml reagent 3 and 50  l of haemolysate were mixed and the absorbance ($A_{HbTotal)}$ read at same wavelength.

## 3.5 Histological Examinations

All tissues were fixed in 10% formalin, the fixed tissues were dehydrated with grades of alcohol beginning from 70% to absolute. They were then cleared with xylene, impregnated and embedded in paraffin wax. Sections were prepared, stained with haematoxylin and eosin (H&E) stain, and then mounted using DPX (Distrene 80 dibutylphthalate Xylene) for light microscopy and photomicrography.

## 3.6 Statistical Analyses

Values obtained were expressed as mean ± standard error of mean. The data were analysed using the Statistical Package for Social Sciences (SPSS) version 17.0 software, and a p-value of $< 0.05$ was considered statistically significant.

# CHAPTER FOUR

# RESULTS

The results of this study are presented in the tables below;

**Table 4.1** shows the mean body weights (initial and final) of the experimental groups at 3 and 5 weeks. There was no statistically significant difference ($p>0.05$) in the means of initial body weights of groups A and B (184.0 ± 4.5g and 188.0 ± 5.7g) at 3 weeks, and 182.0 ± 5.5g and 185.0 ± 4.8g at 5 weeks respectively while the means of the final body weights of groups A and B (198.0 ± 4.2g and 172.0 ± 4.7g) at 3 weeks, and 207.0 ± 5.4g and 153.0 ± 4.0g at 5 weeks respectively showed a statistically significant difference ($p<0.05$)

There was a significant increase in the body weights of the control group while the diabetic group showed a significant decrease in their body weights.

In **Table 4.2,** the mean serum GGT concentration of the diabetic group at 3 and 5 weeks (4.0 ± 0.2iu/l and 4.4 ± 0.2iu/l respectively) were significantly increased ($p<0.001$) when compared to the control group 0.8 ± 0.2iu/l and 0.8 ± 0.1iu/l at 3 and 5 weeks respectively while the mean serum TAC of the diabetic group at 3 and 5 weeks (0.8 ± 0.1mmol/l and 0.6 ± 0.1mmol/l) respectively were significantly decreased ($p<0.001$) when compared to the control 1.6 ± 0.1mmol/l and 1.7 ± 0.1mmol/l at 3 and 5 weeks respectively.

**Table 4.3** shows the correlation coefficient between GGT and TAC at 3 and 5 weeks.

There was a significant ($p<0.001$) negative correlation between GGT and TAC in the experimental groups, $r = -0.707$ and $r = -0.897$ at 3 and 5 weeks respectively.

In **Table 4.4,** the mean Urea, Creatinine, ALT, AST, TC, LDL-C, VLDL-C, TG, FPG and HbA1c values were significantly increased ($p<0.001$) in the diabetic group when compared to the control group while the mean serum HDL-C value of the diabetic group decreased significantly ($p<0.001$) when compared to the control at 3 and 5 weeks of the study.

**TABLE 4.1: BODY WEIGHTS OF NON-DIABETIC AND DIABETIC RATS AT 3 AND 5 WEEKS.**

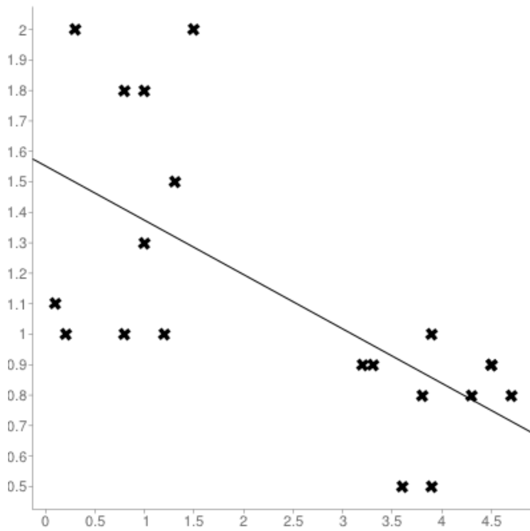
| Group | Initial weight (g) | Final weight (g) | p-value |
|---|---|---|---|
| $A_{(3\ weeks)}$ | 184.0 ± 4.5 | 198.0 ± 4.2 | **>0.05** |
| $B_{(3\ weeks)}$ | 188.0 ± 5.7 | 172.0 ± 4.7 | |
| $A_{(5\ weeks)}$ | 182.0 ± 5.5 | 207.0 ± 5.4 | **>0.05** |
| $B_{(5\ weeks)}$ | 185.0 ± 4.8 | 153.0 ± 4.0 | |

**TABLE 4.2: SERUM GGT AND TAC LEVELS OF NON-DIABETIC AND DIABETIC RATS AT 3 AND 5 WEEKS**

| Group | GGT (iu/l) | TAC (mmol/l) |
|---|---|---|
| $A_{(3\ weeks)}$ | 0.8 ± 0.2 | 1.6 ± 0.1 |
| $B_{(3\ weeks)}$ | 4.0 ± 0.2 | 0.8 ± 0.1 |
| **p-value** | **<0.001** | **<0.001** |
| $A_{(5\ weeks)}$ | 0.8 ± 0.1 | 1.7 ± 0.1 |
| $B_{(5\ weeks)}$ | 4.4 ± 0.2 | 0.6± 0.1 |
| **p-value** | **<0.001** | **<0.001** |

**TABLE 4.3: CORRELATION COEFFICIENT BETWEEN GGT AND TAC OF EXPERIMENTAL GROUPS AT 3 AND 5 WEEKS**

| Parameters | Correlation coefficient (r-value) | p-value |
|---|---|---|
| GGT and $TAC_{(3\ weeks)}$ | -0.707 | **<0.001** |
| GGT and $TAC_{(5\ weeks)}$ | -0.897 | **<0.001** |



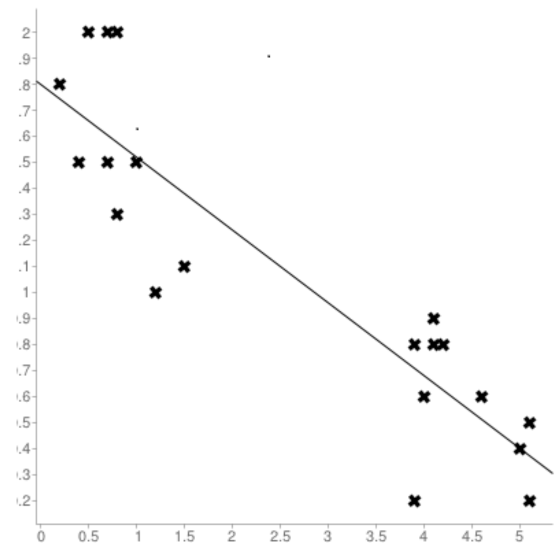



**TABLE 4.4: TEST FOR KIDNEY FUNCTION (UREA & CREATININE), LIVER FUNCTION (ALT & AST), LIPID PROFILE (TC, HDLC, LDLC, VLDLC & TG) AND CARBOHYDRATE METABOLISM (FPG & HbA1c) OF NON-DIABETIC AND DIABETIC RATS AT 3 AND 5 WEEKS**

| Grp | Urea mmol/l | Crea µmol/l | ALT iu/l | AST iu/l | TC mmol/l | HDLC mmol/l | LDLC mmol/l | VLDLC mmol/l | TG mmol/l | FPG mmol/l | HbA1c % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $A_{(3\ wks)}$ | 4.4 ±0.5 | 96.5 ±1.6 | 13.3 ±1.0 | 18.1 ±1.1 | 2.3 ±0.1 | 1.2 ±0.0 | 0.6 ±0.1 | 0.5 ±0.0 | 1.0 ±0.0 | 5.0 ±0.1 | 3.8 ±0.1 |
| $B_{(3\ wks)}$ | 10.4 ±0.3 | 132.6 ±2.0 | 22.4 ±0.8 | 29.2 ±1.5 | 3.8 ±0.1 | 0.6 ±0.0 | 2.3 ±0.1 | 1.0 ±0.0 | 2.3 ±0.0 | 14.4 ±0.1 | 9.1 ±0.5 |
| **p-value** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| $A_{(5\ wks)}$ | 4.8 ±0.5 | 97.4 ±1.4 | 13.0 ±1.1 | 17.3 ±1.1 | 2.4 ±0.1 | 1.3 ±0.0 | 0.7 ±0.0 | 0.5 ±0.0 | 1.1 ±0.0 | 5.1 ±0.1 | 3.9 ±0.1 |
| $B_{(5\ wks)}$ | 10.8 ±0.3 | 138.0 ±2.0 | 24.2 ±0.9 | 32.0 ±1.5 | 3.7 ±0.1 | 0.6 ±0.0 | 2.1 ±0.0 | 1.1 ±0.0 | 2.4 ±0.0 | 13.2 ±0.3 | 10.1 ±0.7 |
| **p-value** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |

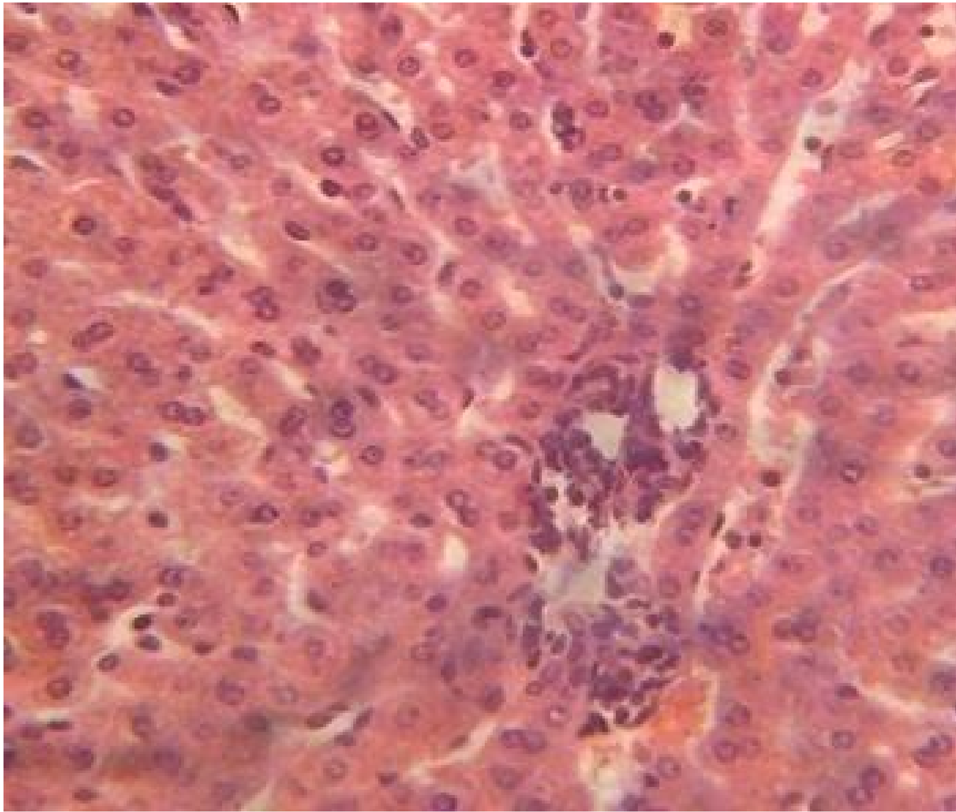


**Fig.1 Liver micrograph of control rat showing normal hepatic cells at 3 and 5 weeks. (H & E stain X400)**

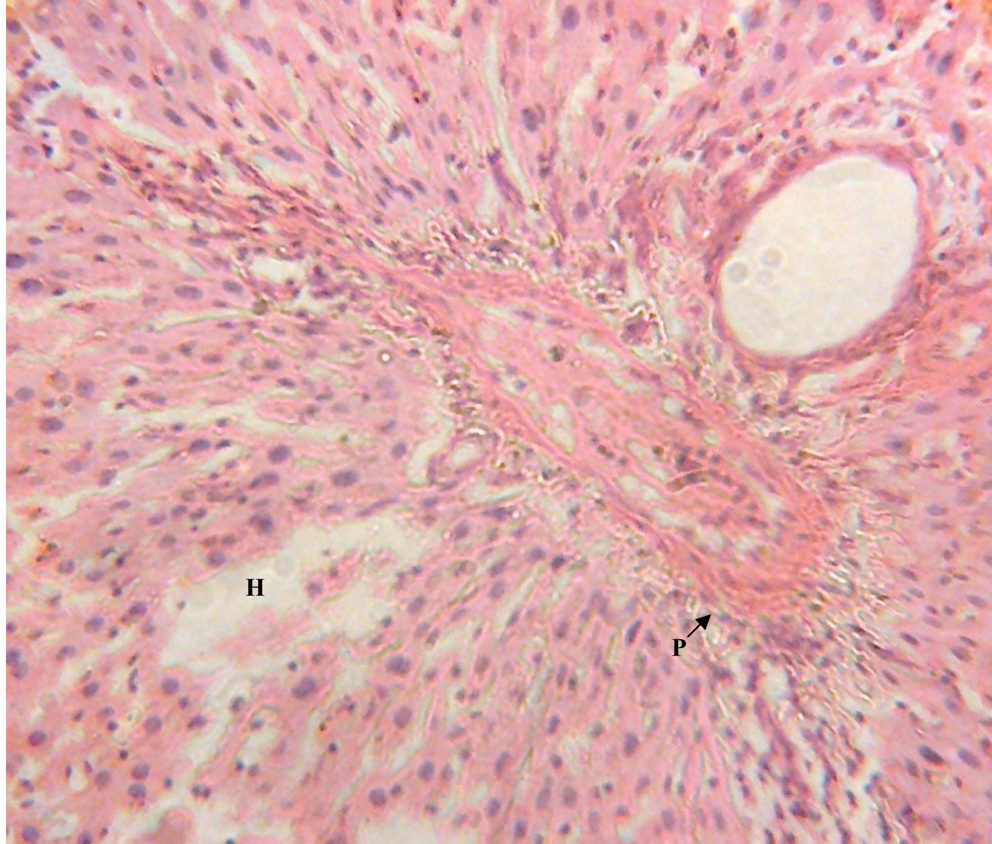



**Fig.2 Liver micrograph of diabetic rat showing mild periportal lymphocytic infiltration(P) and hepatocyte disorientation (H) at 3 weeks. (H & E stain X400)**

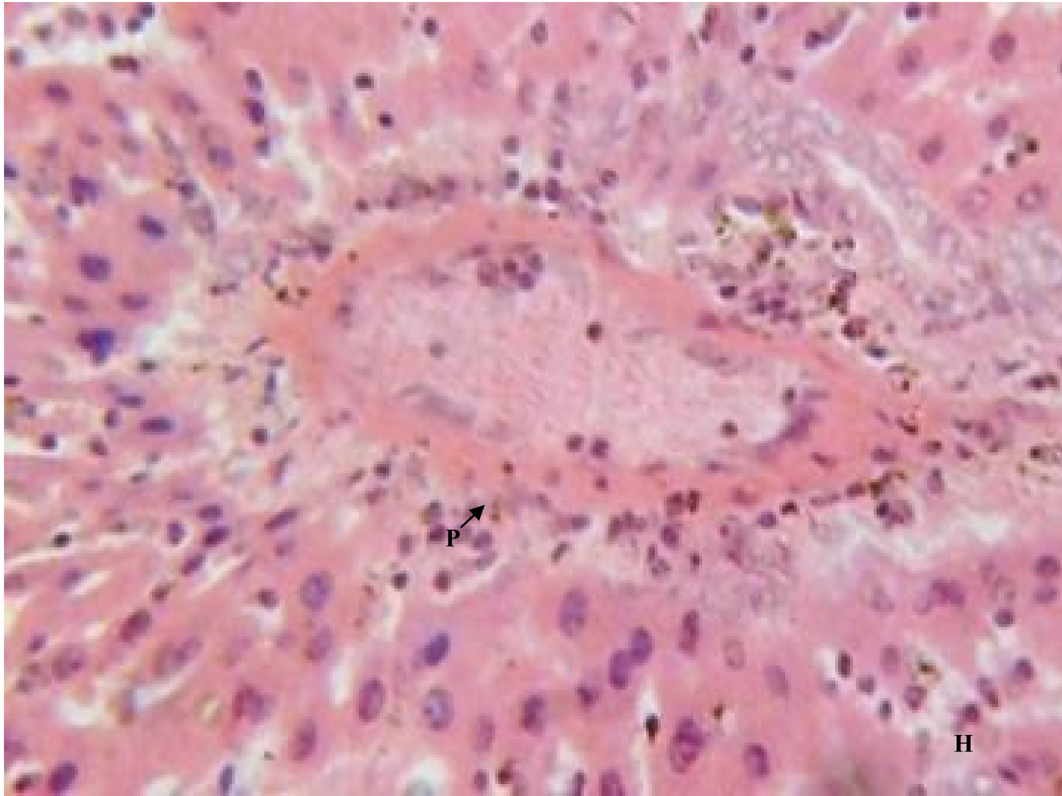



**Fig.3 Liver micrograph of diabetic rat showing moderate periportal lymphocytic infiltration(P) and hepatocyte disorientation (H) at 5 weeks. (H & E stain X400)**

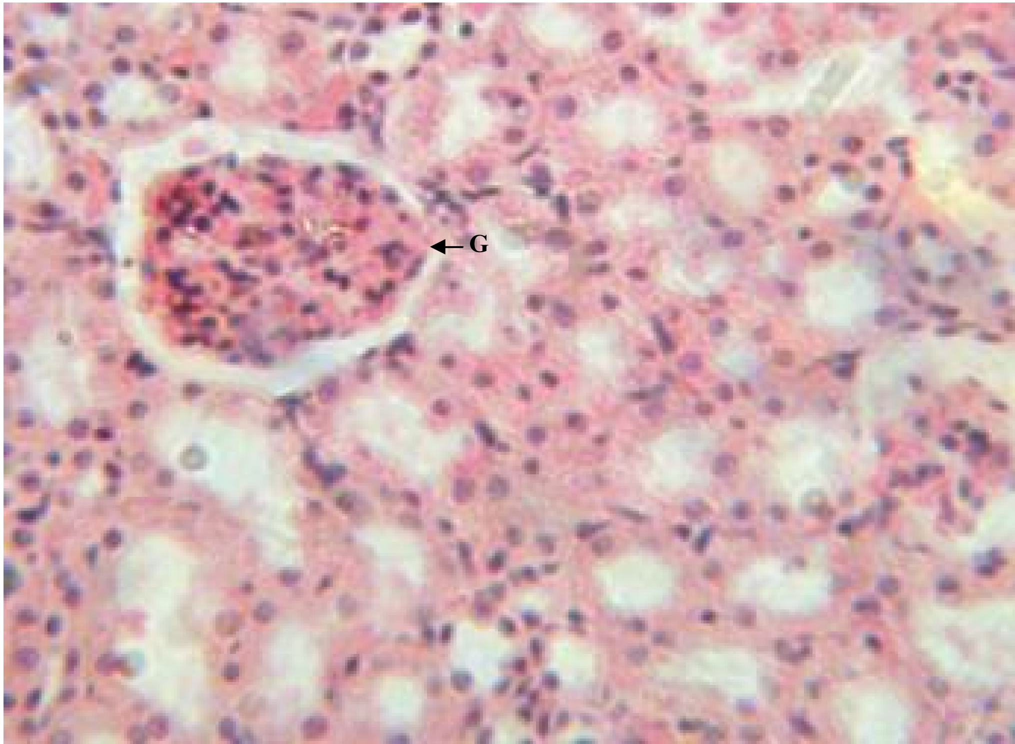



**Fig.4 Kidney micrograph of control rat showing normal glomerular(G) structure at 3 and 5 weeks. (H & E stain X400)**

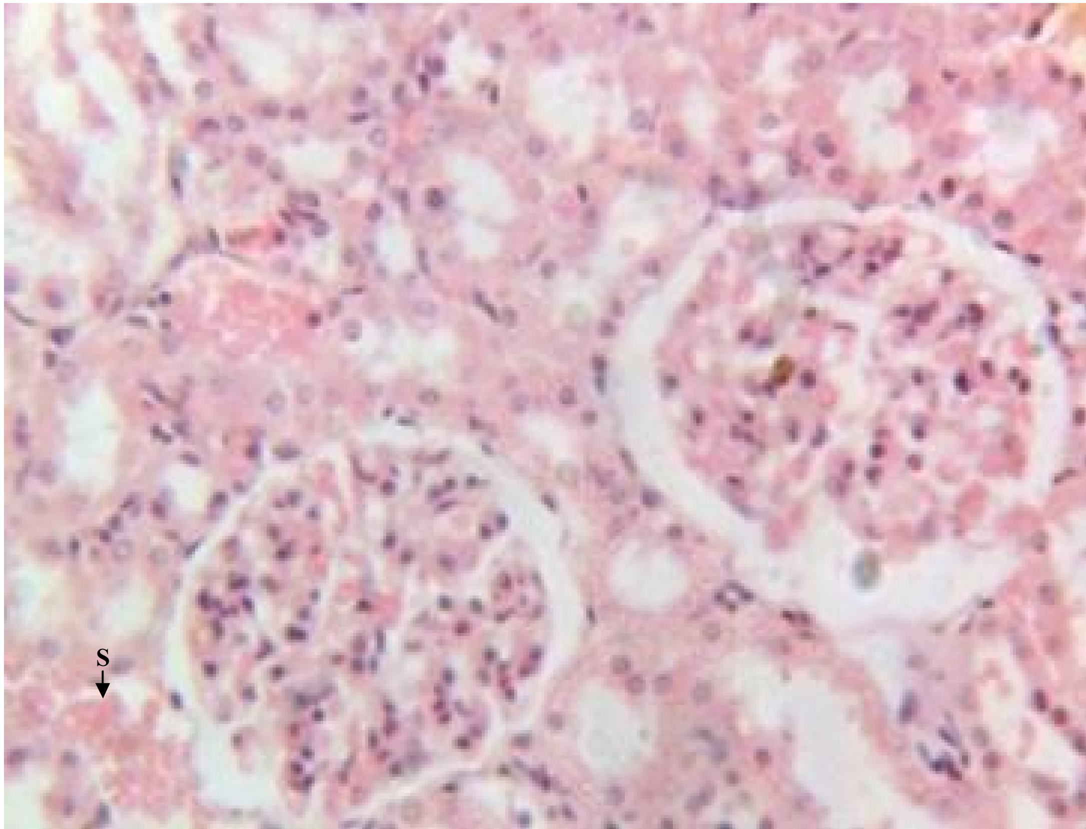



**Fig.5 Kidney micrograph of diabetic rat showing septal haemorrhage(S) at 3 weeks. (H & E stain X400)**

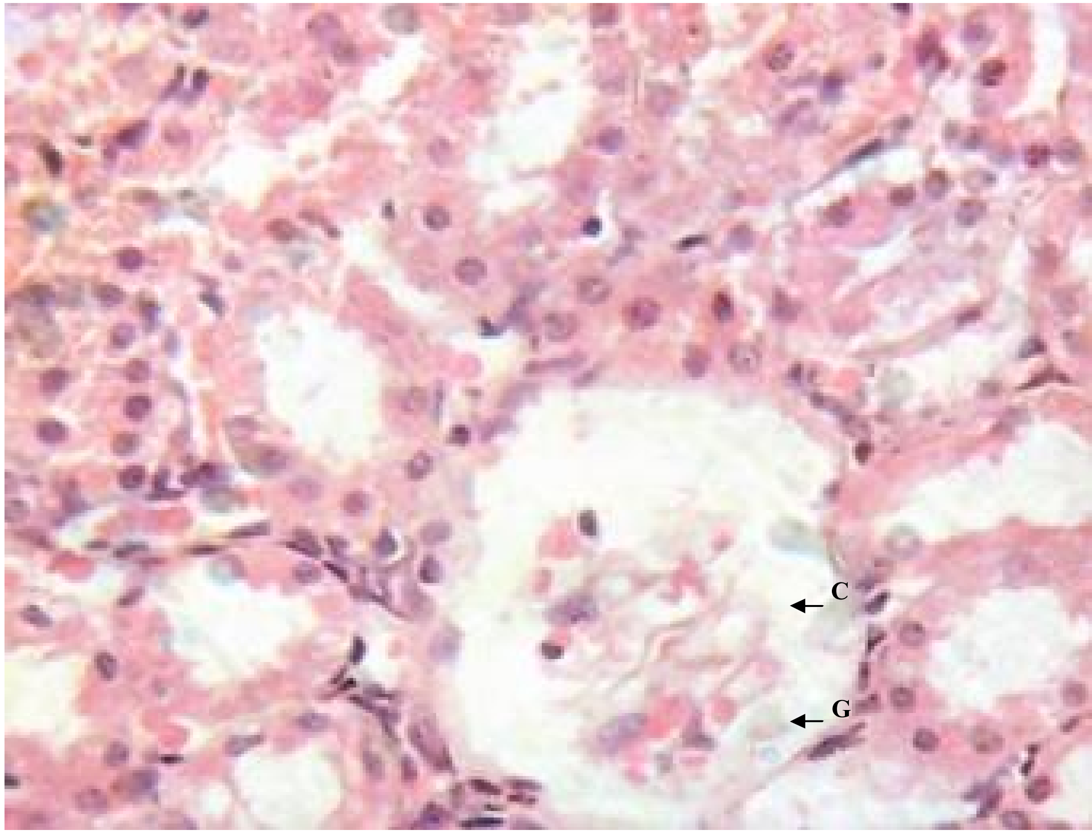



**Fig.6 Kidney micrograph of diabetic rat showing glomerular degeneration(G) and increased capsular space(C) at 5 weeks. (H & E stain X400)**

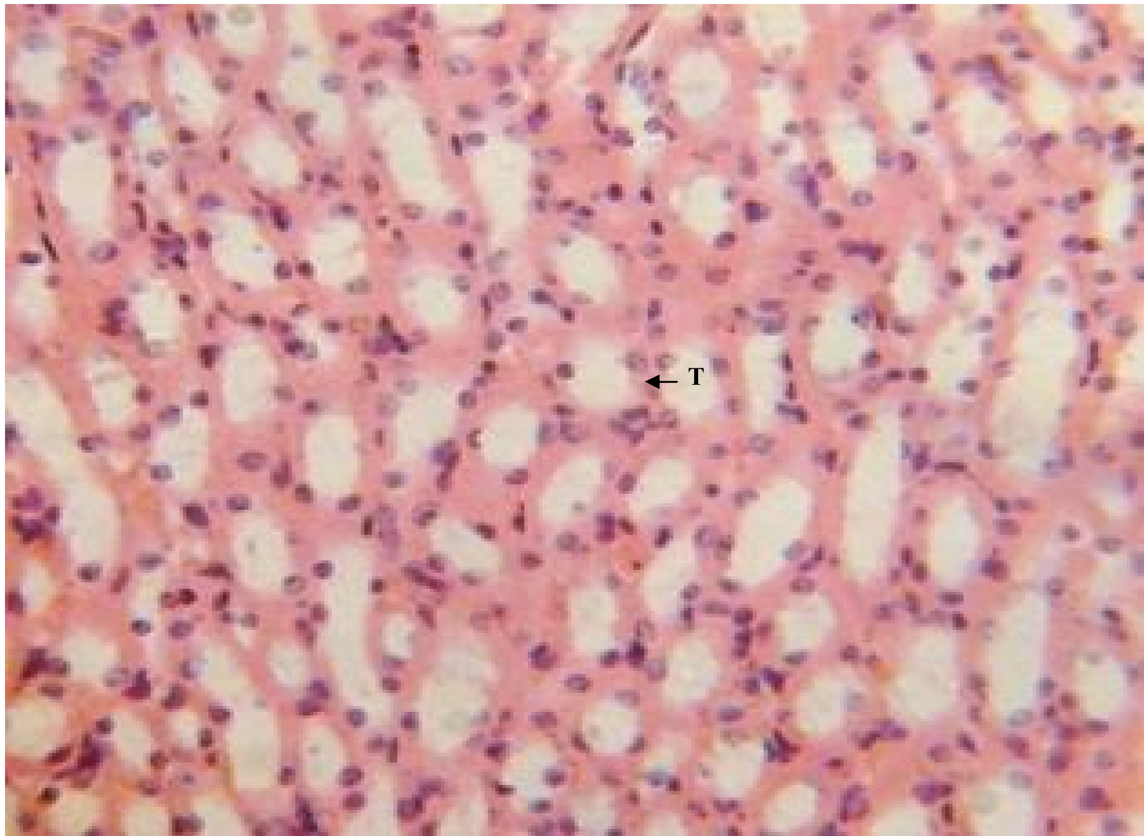



**Fig.7 Kidney micrograph of control rat showing normal tubular(T) structures at 3 and 5 weeks. (H & E stain X400)**

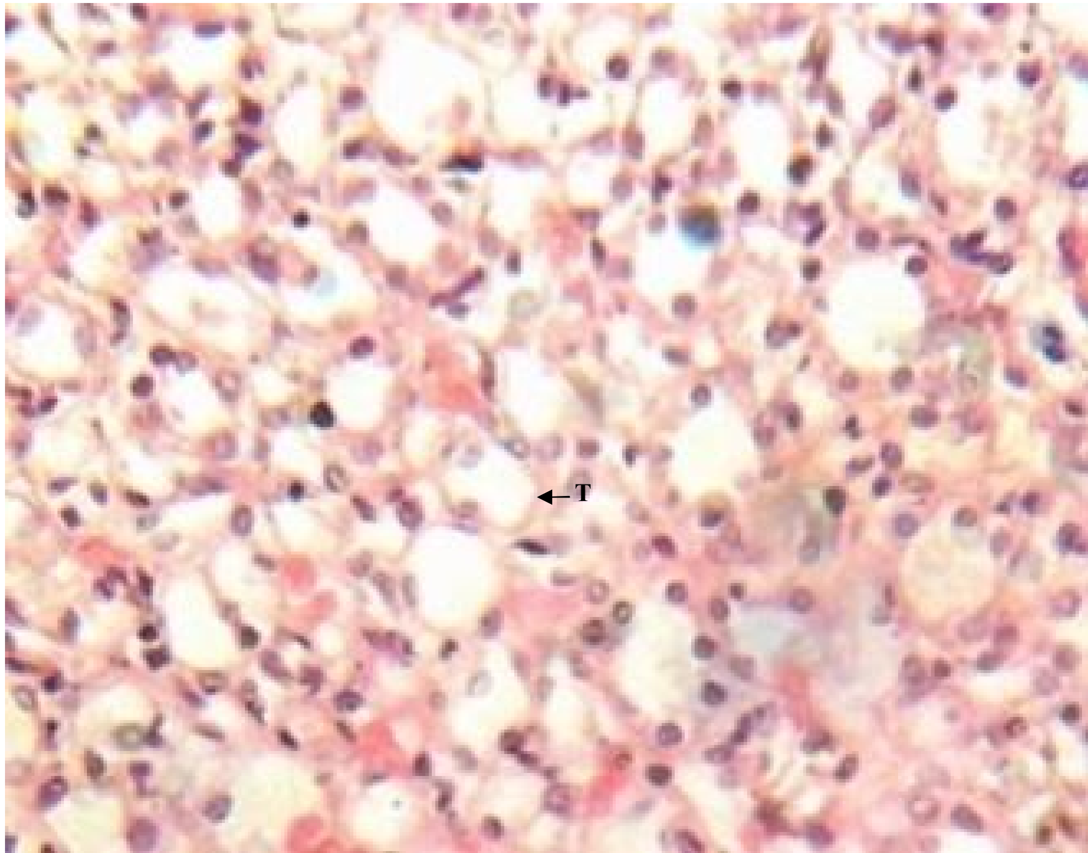



**Fig.8 Kidney micrograph of diabetic rat showing mild tubular damage(T) at 3 weeks. (H & E stain X400)**

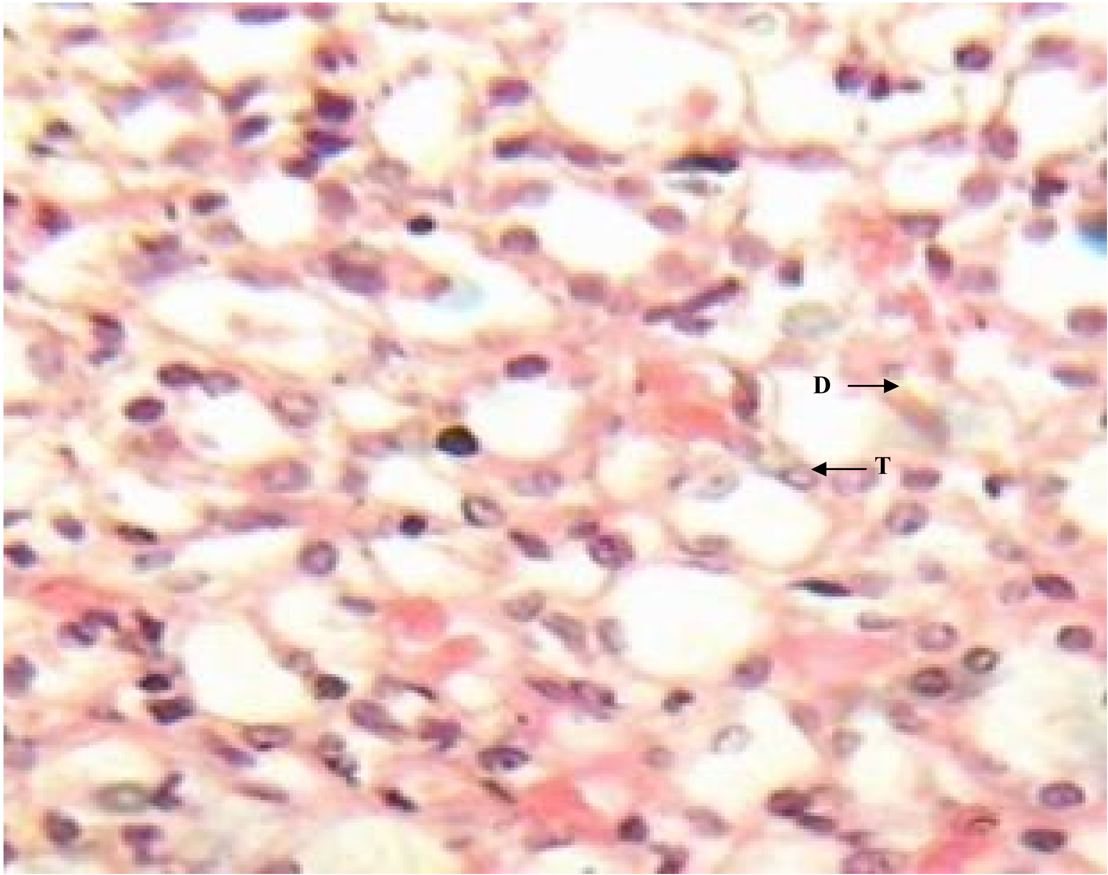



**Fig.9 Kidney micrograph of diabetic rat showing moderate tubular damage(T) and dysplasia(D) at 5 weeks. (H & E stain X400)**

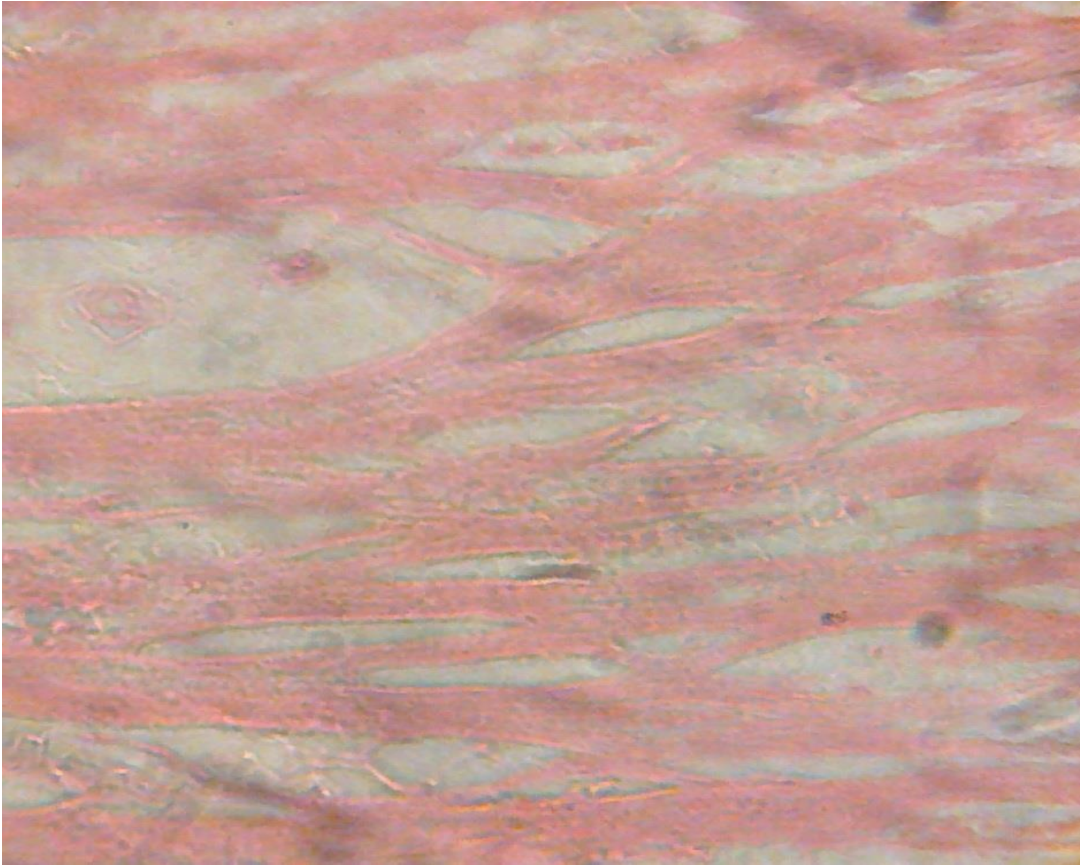


**Fig. 10 Heart micrograph of control rat showing normal cardiac muscle structures at 3 and 5 weeks.**

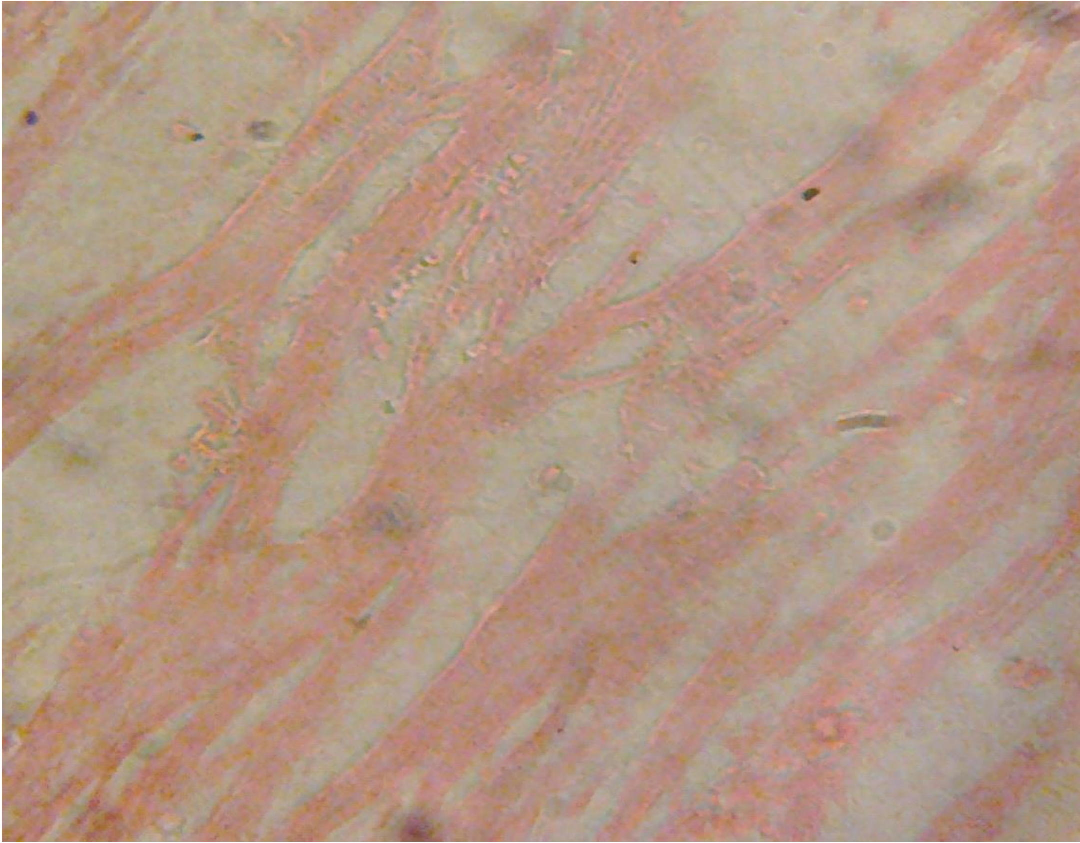


**Fig. 11 Heart section of diabetic rat showing no alterations of the cardiac muscle structures at 3 and 5 weeks.**

# CHAPTER FIVE

# DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Discussions

The present study shows that alloxan injection caused a significant increase in plasma glucose levels in rats in comparison with the control rats. Persistent hyperglycaemia resulted in a number of biochemical and pathological changes in the diabetic rats but the control rats were normoglycaemic and showed no biochemical or pathological changes.

The results of this study are in accordance with the results of Enas, 2011 and Gwarzo *et al,* 2010 who reported that intraperitoneal injection of alloxan induced diabetes. Gidado *et al,* 2009 also reported that diabetes induction in experimental animals caused significant hyperglycaemia.

When rats are injected with alloxan, they provide animal models of insulin dependent diabetes mellitus.

In the present study, the alloxan induced diabetic rats showed significantly higher levels of fasting plasma glucose and lower body weights when compared to the control. This was consistent with earlier reports of Komolafe *et al,* 2009; Murali *et al,* 2002; Urmila and Goyal, 2003. Previous histological studies also supported the present investigation as alloxan was suspected to destroy the B cell islets of Langerhaans according to the reports of Mir, *et al,* 2006; Muthulingam, 2010; Sandhu *et al,* 2000; Szkudelski, 2001 who reported that the pancreas of alloxan or streptozotocin induced diabetic rats showed reduced islet cells and necrosis. The pancreas is especially susceptible to action of alloxan induced free radical damage leading to massive reduction of insulin release, the resultant effect which is insulin deficiency leads to increased blood glucose and a host of other metabolic alterations in the animals.

The weights of the diabetic rats were significantly reduced despite the increase in food and fluid intake in these animals. The weight loss was progressive for five weeks post alloxan administration. The reduction in body weights observed in the diabetic rats compared to the non-diabetic rats may be

due to dehydration and increased catabolism of fats and protein (Hakim *et al,* 1997) as a result of cells inability to metabolize glucose properly (gluconeogenesis from amino acids and body protein). These metabolic derangements eventually result to muscle, tissue wasting and breakdown. This result is in agreement with the work done by Ebuehi *et al,* 2010.

An increase in serum GGT was observed in diabetic rats when compared to normal controls. The results were in accordance with many prospective studies like those of Lee *et al,* 2003; Nakanishi *et al,* 2003; Sharma *et al,* 2010 who reported a strong relationship between GGT concentrations and incident diabetes. This relationship could be as a result of GGT acting as a marker of oxidative stress. Hiderani *et al,* 2005 reported that serum GGT activity may be regarded as a marker of oxidative stress rather than a mere indicator of excessive alcohol consumption or liver dysfunction, and that increased serum GGT may reflect a counter reaction of human bodies against increased oxidative stress. It has been shown that GGT counteracts oxidative stress by breaking down extracellular glutathione and making component amino acids of glutathione available to the cells (Whitfield, 2001).

In the present investigation, diabetic rats exhibited profound decrease in the serum TAC concentration when compared to the control. This could be due to increased ROS generation by mechanisms such as glucose auto-oxidation, polyol pathway and PKC activation.

Evidences suggest that oxidative stress is increased in diabetes resulting from an overproduction of ROS, and decreased efficiency of antioxidant activity in the diabetic rats is indicative of oxidative stress.

The results of this study are in accordance with those of Datta *et al,* 2000; Enas, 2011; Jackson *et al,* 2007; Sait and Hattice, 2009. Houcher *et al,* 2007 reported an increase in antioxidant capacity in alloxan treated rats which he attributed to over expression of antioxidant enzymes in response to glucose-induced oxidative stress.

Serum GGT concentration was higher whereas TAC concentration was lower in diabetic rats compared to the normal control. This negative correlation between GGT and TAC could be

attributed to oxidative stress in diabetes which depletes the antioxidant defense system resulting in a compensatory increase in GGT.

Chronic hyperglycaemia and dyslipidaemia are associated with a variety of metabolic disorders in human and animal diabetes (Handen *et al,* 2008; 2009) causing oxidative stress, depleting the activity of the antioxidant defense system and resulting in elevated levels of ROS (Dahech *et al,* 2011; Handen *et al,* 2011). The inverse association between antioxidants and serum GGT has indicated the possibility that serum GGT might be associated with oxidative stress (Lee *et al,* 2004; Takigawa *et al,* 2008).

In the present study, HbA1c was used as a marker of glycaemic control. The significant increase in HbA1c concentration in the diabetic rats when compared to the control may be attributed to the persistent hyperglycaemia. Persistent hyperglycaemia, uncontrolled or poorly controlled diabetes results to an increased glycosylation of a number of proteins including haemoglobin because the excess glucose in blood reacts with haemoglobin. This result supports the work done by Aruna *et al,* 1999; Muthulingam, 2010; Neeraj *et al,* 2012; Vallejo *et al,* 2000 higher HbA1c levels in diabetic rats.

Our study also showed that alloxan induced diabetes in rats produced alterations in the hepatic structures as well as functions while the control group showed no pathological changes. This was evident from the histological sections which showed mild and moderate periportal lymphocytic infiltrations with sinusoidal dilatations as well as serum elevation of transaminases activities (ALT and AST) which are indices of liver cell damage. These alterations could be attributed to the altered architecture of the hepatocytes, and these subtle membrane changes are sufficient to allow the leakage or passage of intracellular enzymes into blood, hence their increased concentrations in serum. Moreover, cell damage increases permeability causing cytosolic isoenzymes to spill into the interstitium, and from there into the peripheral blood. The pathological changes in liver of alloxan and streptozotocin induced diabetic animals have been previously reported (Herrman *et al,* 1999; Sandhu *et al,* 2000). This is consistent with the reports of Atangwho *et al,* 2007; 2010.

Degirmenchi *et al,* 2002; Isogai *et al,* 1997; Muhammad *et al,* 2009; Muthulingam, 2010; Zhang *et al,* 1995 reported an increased serum levels of ALT and AST in diabetic rats.

Al-shamshi *et al,* 2006; Fadillioglu *et al,* 2007; Ohaeri, 2001 also reported an increased serum ALT and AST in diabetes patients than in the general population.

The results of this study however disagree with the results of Jianpu *et al,* 2010 who reported that serum ALT and AST levels in diabetic rabbits were within normal ranges and Gidado *et al,* 2009 who reported insignificant changes in serum levels of ALT and AST in diabetic rats.

The liver is one of the tissues that bear the brunt of chronic hyperglycaemia, since glucose is freely permeable to its cells (Meyes, 2003). This unrestrained entry, in the presence of excess and sustained glucose in the blood is bound to cause metabolic derangements which would express themselves on the gross architecture of the tissues (Atangwho *et al,* 2010 ).

Present observations on the diabetic rat kidneys showed a progressive damage which increased with duration and severity of hyperglycemia as recorded by Muhammad *et al,* 2009, the resultant renal damage was caused by severe hyperglycemia induced by alloxan.

There were septal haemorrhages, glomerular degeneration, increased capsular space with tubular epithelial damage and dysplasia as observed in kidney sections of the diabetic rats. These findings are in agreement with the findings of Kim *et al,* 2008; Muhammad *et al,* 2009; Renno *et al,* 2008 who showed tubular epithelial changes and enlargement of lining of cells of the tubules.

The structural changes in the kidneys could be attributed to altered metabolism in diabetes (Rasch, 1979) and subsequent effects on the increased renal threshold for hyperglycemia (Mir *et al,* 2006). Moreover, glucose overload damage the mesangial cells in the renal glomerulus through oxidative stress (Brownlee, 2001). In the diabetic kidney, enhanced glucose uptake occurs in many cell populations including glomerular epithelial cells, mesangial cells and proximal tubular epithelial cells leading to the excessive production of intracellular ROS, making these cells particularly susceptible to diabetic milieu (Forbes *et al,* 2008).

Sandhu *et al,* 2000 reported that diabetic dogs present with degeneration of glomeruli and tubular epithelium along with the presence of hyaline casts, mildly sclerotic glomerulus and coagulative necrosis of tubular epithelium.

Bulut *et al,* 2001 reported that glomerular capillaries entirely fill the renal corpuscle along with mesangial cell proliferation and hypertrophy in alloxan-induced diabetic rabbits.

Eze, 2012 reported some degeneration of glomeruli with presence of tubular casts and signs of chronic inflammation in diabetic wistar rats. Peter *et al,* 2006 had previously reported a damaging effect of diabetes in the glomerulus, thereby affecting Glomerular Filtration Rate (GFR). Zafar *et al,* 2009 also reported that streptozotocin-induced diabetic rats showed some functional and morphological changes in the kidney.

These morphological abnormalities in the kidney of diabetic rats were associated with significant elevations in serum urea and creatinine levels, indicating impaired renal function of the diabetic animals.

The results are consistent with those of Eze, 2012; Neeraj *et al,* 2012; Shah *et al,* 2008 who reported elevated serum urea and creatinine levels as a result of renal damage in diabetic rats.

The histological sections of the hearts of diabetic as well as non-diabetic rats showed normal architecture of the myocardium for the study period. This is consistent with the work done by Kita *et al,* 1991 who reported short term metabolic disorders in diabetic rat heart with histopathological changes occurring later. The results disagree with those of Komolafe *et al,* 2009 who reported architectural alterations in myocardium and microanatomy of cardiovascular structures of diabetic animals. Maghrani *et al,* 2004 and Motta *et al,* 2001 also reported that diabetes is associated with profound alterations in biochemical and normal histology leading to an increased coronary heart disease.

In the present study, serum lipid profile was used to assess the risk of development of cardiovascular disease which recorded elevated total cholesterol, LDL-C, VLDL-C and triglyceride levels whereas

HDL-C levels decreased in alloxan-induced diabetic rats which is associated with cardiovascular diseases as seen in diabetes. The abnormal high levels of serum lipids in diabetes is mainly due to increased activity of hormone sensitive lipase in insulin deficiency resulting in enhanced lipolysis and mobilization of free fatty acids from the peripheral depots and adipose due to underutilization of glucose. Some of the excess fatty acid produced is then metabolized to acetyl co A which is used in the synthesis of cholesterol in the liver, thus increasing cholesterol levels in diabetes. On the other hand, glucagon, catecholamines and other hormones may also enhance lipolysis. Therefore, the uninhibited action of lipolytic hormones on the fat depots may be responsible for this increase.

The lack of insulin and elevations of counter regulatory hormones lead to activation of enzyme (hormone-sensitive lipase) that stimulate lipolysis and enhance release of free fatty acids from adipose tissue (Rotimi *et al,* 2011; Subbiah *et al,* 2006). The fatty acids from adipose tissues are mobilized for energy purpose, and excess fatty acids are accumulated in the liver, which are converted to triglycerides (Suryawanshi *et al,* 2006).

The high levels of LDL-C may be attributed to diminished levels of LDL receptors resulting in increased circulating LDL particles (Suryawanshi *et al,* 2006). The decreased serum HDL-C levels in the present study may enhance CVD risk since HDL-C function is to remove cholesterol atheromas within arteries and transport them back to the liver for excretion and re-utilization.

The results support the work of Enas, 2011; Gidado *et al,* 2009; Komolafe *et al,* 2009; Mendez and Balderas, 2001; Nandhakuma *et al,* 2007; Sait and Hatice, 2009; Shah *et al,* 2008; Shirwaikar *et al,* 2004 who reported marked increase in cholesterol, triglycerides, LDL-C, VLDL-C and decreased HDL-C in diabetic rats when compared to non-diabetic rats.

However, Jos *et al,* 1990 reported no correlation between diabetes mellitus and lipids in the study conducted with diabetes patients.

## 5.2 Conclusions

From our study, alloxan-induced diabetes caused an elevation of serum GGT and decreased total antioxidant levels which suggest that GGT and TAC may be used as reliable markers of oxidative stress in diabetes mellitus.

The pathologic lesions and various alterations in the biochemical markers of liver and kidney functions resulting in their structural and functional abnormalities may be regarded as oxidative stress-induced diabetic complications in these organs.

It may also be concluded that the effect of diabetes on the heart results in metabolic and biochemical disorders which may eventually lead to morphological changes at a later stage of the disease.

## 5.3 Recommendations for Further Work

With respect to this study and related ones, it is recommended that further work be done on the following;

a. Examine the association of serum GGT and incidence of diabetes.

b. Assay of individual antioxidants in diabetes.

c. Long term effect of diabetes on the histology of the heart of diabetic rats.

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**APPENDIX 1a**

**RAW DATA OF NON-DIABETIC RATS AT 3 WEEKS**

| S/N | Urea (mmol/l) | Crea (µmol/l) | ALT (iu/l) | AST (iu/l) | TC (mmol/l) | HDLC (mmol/l) | LDLC (mmol/l) | VLDLC (mmol/l) | TG (mmol/l) | FPG (mmol/l) | HbA1c (%) | GGT (iu/l) | TAC (mmol/l) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2.4 | 89.4 | 16.0 | 22.0 | 2.2 | 1.2 | 0.5 | 0.5 | 1.2 | 5.2 | 4.1 | 0.2 | 1.0 |
| 2 | 2.1 | 98.3 | 12.0 | 17.0 | 2.4 | 1.1 | 0.8 | 0.5 | 1.0 | 4.7 | 3.6 | 0.1 | 1.1 |
| 3 | 4.8 | 103.7 | 9.0 | 14.0 | 2.3 | 1.4 | 0.5 | 0.4 | 0.9 | 4.5 | 3.4 | 1.5 | 2.0 |
| 4 | 5.5 | 101.9 | 16.0 | 22.0 | 2.6 | 1.2 | 0.9 | 0.5 | 1.0 | 4.7 | 3.8 | 0.3 | 2.0 |
| 5 | 6.9 | 99.2 | 12.0 | 14.0 | 2.2 | 1.1 | 0.7 | 0.4 | 0.9 | 5.2 | 4.0 | 1.3 | 1.5 |
| 6 | 5.2 | 90.3 | 9.0 | 14.0 | 2.4 | 1.3 | 0.6 | 0.5 | 1.2 | 5.4 | 4.2 | 1.2 | 1.0 |
| 7 | 3.1 | 99.2 | 12.0 | 14.0 | 2.6 | 1.3 | 0.8 | 0.5 | 1.0 | 5.4 | 3.8 | 0.8 | 1.0 |
| 8 | 6.2 | 93.9 | 19.0 | 22.0 | 2.1 | 1.4 | 0.2 | 0.5 | 1.0 | 5.2 | 3.7 | 1.0 | 1.3 |
| 9 | 3.5 | 90.3 | 16.0 | 17.0 | 2.4 | 1.2 | 0.8 | 0.4 | 0.9 | 4.7 | 3.6 | 0.8 | 1.8 |
| 10 | 4.2 | 99.2 | 12.0 | 22.0 | 2.2 | 1.1 | 0.6 | 0.5 | 1.0 | 5.2 | 4.1 | 1.0 | 1.8 |

**RAW DATA OF NON-DIABETIC RATS AT 5 WEEKS**

| S/N | Urea (mmol/l) | Crea (µmol/l) | ALT (iu/l) | AST (iu/l) | TC (mmol/l) | HDLC (mmol/l) | LDLC (mmol/l) | VLDLC (mmol/l) | TG (mmol/l) | FPG (mmol/l) | HbA1c (%) | GGT (iu/l) | TAC (mmol/l) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 3.1 | 100.6 | 16.0 | 22.0 | 2.5 | 1.4 | 0.6 | 0.5 | 1.2 | 5.2 | 3.9 | 0.4 | 1.5 |
| 2 | 2.8 | 89.8 | 19.0 | 22.0 | 2.3 | 1.3 | 0.5 | 0.5 | 1.2 | 4.7 | 3.7 | 0.2 | 1.8 |
| 3 | 4.2 | 98.8 | 9.0 | 14.0 | 2.2 | 1.2 | 0.5 | 0.5 | 1.0 | 4.5 | 3.8 | 0.7 | 2.0 |
| 4 | 5.9 | 104.2 | 16.0 | 17.0 | 2.5 | 1.2 | 0.8 | 0.5 | 1.0 | 4.7 | 4.1 | 1.5 | 1.1 |
| 5 | 7.3 | 98.8 | 12.0 | 17.0 | 2.5 | 1.1 | 0.9 | 0.5 | 0.9 | 5.2 | 4.1 | 0.8 | 1.3 |
| 6 | 6.2 | 97.9 | 12.0 | 14.0 | 2.5 | 1.3 | 0.7 | 0.5 | 1.0 | 5.4 | 4.4 | 1.0 | 1.5 |
| 7 | 3.5 | 98.8 | 16.0 | 22.0 | 2.4 | 1.2 | 0.6 | 0.6 | 1.3 | 5.4 | 3.8 | 0.8 | 2.0 |
| 8 | 5.9 | 97.0 | 9.0 | 14.0 | 2.1 | 1.4 | 0.2 | 0.5 | 1.2 | 5.2 | 3.6 | 0.5 | 2.0 |
| 9 | 3.1 | 97.9 | 12.0 | 17.0 | 2.7 | 1.3 | 0.9 | 0.5 | 1.0 | 4.7 | 4.1 | 1.2 | 1.0 |
| 10 | 6.2 | 89.8 | 9.0 | 14.0 | 2.5 | 1.2 | 0.8 | 0.5 | 0.9 | 5.2 | 3.8 | 0.7 | 1.5 |

## APPENDIX 1b

### RAW DATA OF DIABETIC RATS AT 3 WEEKS

| S/N | Urea (mmol/l) | Crea (µmol/l) | ALT (iu/l) | AST (iu/l) | TC (mmol/l) | HDLC (mmol/l) | LDLC (mmol/l) | VLDLC (mmol/l) | TG (mmol/l) | FPG (mmol/l) | HbA1c (%) | GGT (iu/l) | TAC (mmol/l) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 10.4 | 121.6 | 23.0 | 32.0 | 4.0 | 0.6 | 2.4 | 1.0 | 2.2 | 13.3 | 10.9 | 3.6 | 0.5 |
| 2 | 10.4 | 129.6 | 19.0 | 38.0 | 4.2 | 0.6 | 2.6 | 1.0 | 2.3 | 12.8 | 8.1 | 4.7 | 0.8 |
| 3 | 9.7 | 135.9 | 19.0 | 26.0 | 4.0 | 0.5 | 2.5 | 1.0 | 2.3 | 14.4 | 12.0 | 4.5 | 0.9 |
| 4 | 12.9 | 130.5 | 26.0 | 32.0 | 3.7 | 0.6 | 3.0 | 1.1 | 2.4 | 13.1 | 8.0 | 3.9 | 0.5 |
| 5 | 10.0 | 142.1 | 23.0 | 26.0 | 3.5 | 0.7 | 1.8 | 1.0 | 2.3 | 15.3 | 7.8 | 3.3 | 0.9 |
| 6 | 11.1 | 129.6 | 23.0 | 26.0 | 3.6 | 0.6 | 2.0 | 1.0 | 2.3 | 13.7 | 7.9 | 3.8 | 0.8 |
| 7 | 10.0 | 134.4 | 19.0 | 22.0 | 4.1 | 0.5 | 2.5 | 1.1 | 2.4 | 16.9 | 9.0 | 3.2 | 0.9 |
| 8 | 10.4 | 130.5 | 23.0 | 32.0 | 3.6 | 0.6 | 2.0 | 1.0 | 2.2 | 16.2 | 8.4 | 4.3 | 0.8 |
| 9 | 10.0 | 142.1 | 26.0 | 26.0 | 3.6 | 0.7 | 1.9 | 1.0 | 2.3 | 14.2 | 10.9 | 3.9 | 1.0 |
| 10 | 9.3 | 129.6 | 23.0 | 32.0 | 3.4 | 0.5 | 1.9 | 1.0 | 2.2 | 13.7 | 8.0 | 4.5 | 0.9 |

### RAW DATA OF DIABETIC RATS AT 5 WEEKS

| S/N | Urea (mmol/l) | Crea (µmol/l) | ALT (iu/l) | AST (iu/l) | TC (mmol/l) | HDLC (mmol/l) | LDLC (mmol/l) | VLDLC (mmol/l) | TG (mmol/l) | FPG (mmol/l) | HbA1c (%) | GGT (iu/l) | TAC (mmol/l) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 10.4 | 132.3 | 23.0 | 32.0 | 3.9 | 0.6 | 2.2 | 1.1 | 2.4 | 11.9 | 9.4 | 5.1 | 0.2 |
| 2 | 11.4 | 134.1 | 26.0 | 38.0 | 4.0 | 0.7 | 2.3 | 1.0 | 2.3 | 12.1 | 13.2 | 5.0 | 0.4 |
| 3 | 11.1 | 148.4 | 23.0 | 32.0 | 3.9 | 0.5 | 2.3 | 1.1 | 2.5 | 13.0 | 14.6 | 5.1 | 0.5 |
| 4 | 12.9 | 132.3 | 30.0 | 38.0 | 3.6 | 0.5 | 2.1 | 1.0 | 2.3 | 12.3 | 10.3 | 4.6 | 0.6 |
| 5 | 10.0 | 142.9 | 26.0 | 26.0 | 3.5 | 0.6 | 1.8 | 1.1 | 2.5 | 13.6 | 8.1 | 4.1 | 0.8 |
| 6 | 9.7 | 134.1 | 23.0 | 32.0 | 3.5 | 0.6 | 1.8 | 1.1 | 2.5 | 12.7 | 9.4 | 4.2 | 0.8 |
| 7 | 11.4 | 143.9 | 19.0 | 26.0 | 4.2 | 0.7 | 2.4 | 1.1 | 2.4 | 15.3 | 8.1 | 3.9 | 0.8 |
| 8 | 10.4 | 134.1 | 23.0 | 38.0 | 3.6 | 0.5 | 2.0 | 1.1 | 2.5 | 15.1 | 8.0 | 4.0 | 0.6 |
| 9 | 10.0 | 134.1 | 26.0 | 26.0 | 3.7 | 0.5 | 2.1 | 1.1 | 2.4 | 13.0 | 10.3 | 4.1 | 0.9 |
| 10 | 11.1 | 143.9 | 23.0 | 32.0 | 3.4 | 0.6 | 2.0 | 1.1 | 2.5 | 13.0 | 9.4 | 3.9 | 0.2 |

## APPENDIX 11
## REAGENTS

1. Glucose assay kit (Randox).
2. Total cholesterol TC assay kit (Randox).
3. High Density Lipoprotein Cholesterol HDL-C assay kit (Randox).
4. Triglycerides TG assay kit (Randox).
5. Total Antioxidant Capacity:
    a. Sodium Phosphate Buffer pH 7.4 (10mmol/l)
    b. Sodium Benzoate (10mmol/l)
    c. Sodium Hydroxide (50mmol/l)
    d. EDTA (2mmol/l)
    e. Iron-Ammonium Sulphate $Fe(NH_4)SO_4$ (2mmol/l)
    f. Iron-EDTA complex [equal vol. $Fe(NH_4)SO_4$ and EDTA]
    g. Acetic Acid (20%)
    h. Thiobarbituric Acid
    i. Uric Acid (1mmol/l)
6. Glycated Haemoglobin (HbA1c):
    a. R1: Potassium Phthalate (50mmol/l)
       Sodium Azide (0.95g/l)
       Detergent (5g/l)
    b. R2: Phosphate Buffer pH 6.5 (30mmol/l)
       Sodium Azide (0.95g/l)
    c. R3: Phosphate Buffer pH 6.5 (72mmol/l)
       Sodium Azide (0.95g/l)
7. Creatinine
    a. Picric Acid (0.04M)
    b. Sodium Hydroxide (0.75M)
    c. Sodium Tungstate (0.15M)
    d. Sulphuric Acid (0.66M)

8. Urea
    a. Diacetylmonoxime Reagent (Mixed colour)
        - Diacetylmonoxime 1.34g

- Thiosemicarbazide 0.335g
- Distilled water 1000ml

b. Mixed Acid Reagent

- $FeCl_3.6H_2O$ 0.01g
- 85% $H_3PO_4$ 0.2ml
- Distilled water 200ml
- Conc. $H_2SO_4$ 200ml
- Distilled water to 1000ml

9. Transaminases (Alanine and Aspartate)

a. Phosphate Buffer pH 7.4 0.1M
b. $Na_2HPO_4$ (anhydrous) 11.9g
c. $KH_2PO_4$ (anhydrous) 2.2g
d. Distilled water to 800ml
e. ALT Substrate
f. AST Substrate
g. Colour Reagent (2,4-DNPH)
h. NaOH 0.4N

**APPENDIX III**

**STATISTICAL FORMULAE**

Data obtained were analyzed with statistical package for social sciences (SPSS) Computer software.

The statistical methods used are:

1 Mean ($\bar{x}$) = $\frac{\Sigma \bar{x}}{n}$

Where $\Sigma \bar{x}$ = sum of all the values obtained

n = number of values obtained.

2. Standard deviation (SD) = $\sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$

Where x = Values obtained

$\bar{x}$ = Mean of values obtained

N = Number of values obtained

3. SE ($\bar{x}$) = $S/\sqrt{n}$

Where SE = standard Error

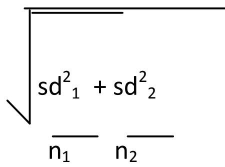
S = Sample standard deviation

$\bar{x}$ = Sample mean

$\sqrt{n}$ = Square root of sample size

4. Student's t - test analysis

$$Tcal = \frac{x_1 - x_2}{\sqrt{\frac{sd^2_1}{n_1} + \frac{sd^2_2}{n_2}}}$$



Where $n_1$ = Number of sample study for control values

$n_2$ = Number of sample study for test

$X_1$ = Mean of control values

$X_2$ = Mean of test values

$SD_1$ = Standard deviation of control values

SD2 = Standard deviation of test values

**GLOSSARY OF ABBREVIATIONS AND THEIR MEANINGS**

| | | |
|---|---|---|
| IPF | - | Insulin Promoter Factor |
| PCOS | - | Polycystic Ovarian Syndrome. |
| GAD | - | Glutamate Decarboxylase |
| DKA | - | Diabetic Ketoacidosis |
| HHA | - | Hyperglycaemic Hyperosmolar State |
| GFR | - | Glomerular Filtration Rate |
| ESRD | - | End Stage Renal Disease |
| GAPDH | - | Glyceraldehyde-3-Phosphate Dehydrogenase |
| GFAT | - | Glutamine:Fructose-6-phosphate Aminotransferase |
| UDP-GlcNAc | - | Uridine Diphosphate-N-Acetylglucosamine |
| AGEs | - | Advanced Glycated End products |
| Gln | - | Glutamine |
| Glu | - | Glutamate |
| NAD(P) | - | Nicotinamide Adenine Dinucleotide (Phosphate) |
| DHAP | - | Dihydroxyacetone Phosphate |
| DAG` | - | Diacylglycerol |
| GR | - | Glutathione Reductase |
| GSSG | - | Oxidized Glutathione |
| PAI-1 | - | Plasminogen Activator Inhibitor-1 |
| TGF | - | Transforming Growth Factor |
| eNOS | - | Endothelial Nitric Oxide Synthase |
| ET-1 | - | Endothelin-1 |

| | | |
|---|---|---|
| RAGE | - | Receptor for Advanced Glycated End Product |
| NF | - | Nuclear Factor |
| NO | - | Nitric Oxide |
| LDLC | - | Low Density Lipoprotein Cholesterol |
| HDLC | - | High Density Lipoprotein Cholesterol |
| ARBs | - | Angiotensin Receptor Blockers |
| ACE | - | Angiotensin Converting Enzyme |
| MAPK | - | Mitogen-Activated Protein Kinase |
| JNK/SAPK | - | c-Jun $NH_2$-Terminal Kinase/Stress-Activated Protein Kinase |
| ADA | - | American Diabetes Association |
| IDF | - | International Diabetes Federation |
| PKC | - | Protein Kinase C |
| GGT | - | Gamma Glutamyl Transferase |
| TAC | - | Total Antioxidant Capacity |
| ROS | - | Reactive Oxygen Species |
| GSH | - | Reduced Glutathione |
| SOD | - | Superoxide Dismutase |
| GPx | - | Glutathione Peroxidase |
| IGT | - | Impaired Glucose Tolerance |
| GLUT | - | Glucose Transporter |
| ACTH | - | Adrenocorticotrophic Hormone |
| TNF- | - | Tumor Necrosis Factor |
| IFG | - | Impaired Fasting Glucose |

| | | |
|---|---|---|
| CVD | - | Cardiovascular Disease |
| DNA | - | Deoxyribonucleic acid |
| Na-K ATPase | - | Sodium-Potassium Adenine Triphosphatase |
| GIP | - | Glucose-Dependent Insulinotropic Peptide |
| GLP-1 | - | Glucagon-Like Peptide 1 |
| DPP-IV | - | Dipeptidyl Peptidase- IV |
| IRS-1 | - | Insulin Receptor Substrate 1 |
| IDDM | - | Insulin Dependent Diabetes Mellitus |
| LADA | - | Latent Autoimmune Diabetes in Adult |
| WHO | - | Word Health Organization |
| NIDDM | - | Non-Insulin Dependent Diabetes Mellitus |
| CNS | - | Central Nervous System |
| MODY | - | Maturity-Onset Diabetes of the Young |
| HNTF | - | Hepatic Nuclear Transcription Factor |
| RNS | - | Reactive Nitrogen Species |
| HO | - | Heme Oxygenase |
| Trx | - | Thioredoxin |
| $^{\cdot}O_2^-$ | - | Superoxide Anion |
| OH | - | Hydroxyl Radical |
| $ONOO^-$ | - | Peroxynitrte |
| EDNO | - | Endothelial Derived Nitric Oxide |
| MMPs | - | Matrix Metalloproteinases |
| VSM | - | Vascular Smooth Muscle |

| | | |
|---|---|---|
| Ox-LDL | - | Oxidation of Low Density Lipoprotein |
| MCP-1 | - | Monocyte Chemoattractant Protein 1 |
| TGF | - | Transforming Growth Factor |
| AP-1 | - | Activator Protein-1 |
| ECM | - | Extracellular Matrix |
| VCAM-1 | - | Vascular Cell Adhesion Molecule 1 |
| RAS | - | Renin-Angiotensin System |
| $AT_1$ | - | Angiotensin II Type 1 |
| SERCA | - | Sarcoplasmic Reticulum Calcium |
| ICAM | - | Intercellular Adhesion Molecule |
| IL-1 | - | Interleukin 1 |
| TNF | - | Tumor Necrosis Factor |
| LOH | - | Lipid Hydroxide |
| LOOH | - | Lipid Peroxide |
| TXnip | - | Thioredoxin- Interacting Protein |
| DNPH | - | Dinitrophenylhydrazine |
| TBARS | - | Thiobarbituric Acid Reactive Substances |