`

EFFECTS OF GILBENCLAMIDE (GLANIL) ON SOME BIOCHEMICAL PARAMETERS OF ALLOXAN INDUCED DIABETIC RATS

BY

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CERTIFICATION

This is to certify that this thesis titled “Effects of gilbenclamide (glanil) on some Biochemical parameters of alloxan induced diabetic rats” presented by Chukwuelobe, Lilian Chinwe for an M.sc degree in Applied Biochemistry of Nnamdi Azikiwe University, is a bonafide record of research work done under my supervision. The work embodied in this thesis is original and has not been submitted in part or full, for any other degree or diploma of this or any other University.

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(Supervisor)

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(HEAD OF DEPARTMENT)

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External Examiner

DEDICATION

This project is dedicated to my family, brothers and sisters and my friends and well wishers.

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ABSTRACT

The effect of gilbenclamide (glanil) was studied on alloxan induced diabetic *Sprague dawley* rats. The levels of alkaline phosphatase activity, amylase activity, creatinine, total protein, sodium and chloride concentration were determined spectrophotometrically in the serum, kidney, and liver, and blood glucose was determined using glucometer. Establishment of diabetes mellitus in the test animals after eight days was confirmed when the mean fasting blood glucose level in the induced diabetic rats(291.00± 17.04mg/100ml) was significantly higher ($p<0.05$) than that of the control rats (100.20 ±3.06mg/100ml). There was a return to normal fasting blood glucose level of the glanil treated rats (88.25 ± 10.80mg/100ml) after three days treatment. Serum alkaline phosphatase activity of the control, diabetic and glanil treated rats were(3.12 ± 0.44, 6.50 ± 0.50, 8.17 ±2.10) µM/min/ml respectively. There was a significant increase ($p<0.05$) in the serum alkaline phosphatase activity of the glanil treated and diabetic rats. Serum amylase activity of the control, diabetic and treated rats were observed as follows (60.49 ±19.23, 140.78 ±8.15,80.87 ±23.34)IU/L respectively. The was a significant increase($p<0.05$) in serum amylase of the diabetic rats compared to the treated. Serum creatinine of the control, diabetic and treated rats were (472.28 ±69.13, 619.48 ±76.41,203.23 ±57.72) µmol/L respectively. There was a significant increase ($p< 0.05$) in the serum creatinine of the diabetic rats compared to the treated. Kidney total protein concentration of the control, diabetic and glanil treated rats were (5.47 ±3.18, 5.94 ±2.45, 5.89 ±2.17) mg/ml respectively. The liver sodium concentration of the control, diabetic, and treated rats were (156.55 ±29.97, 86.18 ±32.80, 82.41 ±20.88) mEq/L respectively. Serum chloride concentration of the control, diabetic, and treated rats were (96.58 ±1.13, 113.27 ±11.63, 70.99 ±16.34) mEq/L respectively. Serum chloride concentration of diabetic rats were significantly increased at ($p < 0.05$) compared to the treated and control rats. The results showed a reversal to normal fasting blood glucose level of the treated rats and glanil was able to alleviate some of the symptoms of diabetes mellitus. Routine use of glucose level, alkaline phosphatase activity , creatinine concentration, amylase activity, total protein, sodium and chloride concentration measurements be used to monitor diabetic status.

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Abbreviation

Some abbreviations include

L-ALP	Liver alkaline phosphatase
B-ALP	Bone alkaline phosphatase
ALP	Alkaline phosphatase
A	Alpha
G	Gram
<	Less than.
Mg	milligram

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1.0 CHAPTER ONE

1.1 INTRODUCTION

Diabetes mellitus (DM) is the most common type of diabetes with clinical manifestation of hyperglycemia which results in chronic metabolic disorder, of carbohydrates , proteins and fats (Harris *et al* 1987). Diabetes mellitus arises from a deficiency of insulin and can be produced in animals by surgical removal of much of the pancreas or by chemical destruction of the β-cells of islets of Langergahans, such chemicals as alloxan, or anti-insulin antibodies could result to such a destruction of β-cells.

Hyperglycemia results from deficiency of insulin response (Harris *et al* 1987). Other characteristic features include polydipsia, glycosuria, polyuria, ketoacidosis, reduced body weight, ketonuria and hyperosmolaemia. In prolonged diabetes, it is usually complicated with degenerative diseases of blood vessels, retina, kidney, nervous tissues and eventually death if not treated (Munana 1995)

Langerhans (1869) discovered the cells in which insulin is produced in the pancreas which he named the islets of Langerhans. Also, several studies carried out by Von and Minkowski (1889) on dogs by removing the pancreas confirmed that diabetes is associated with pancreatic functioning. Bantan (1921) isolated insulin from the pancreas and proved that pancreas excretes an anti- diabetic substance which enters the blood systems and stabilizes the blood glucose levels.

Joslin *et al* (1946) proposed the treatment of diabetes with insulin administration and this has proved the most efficacious treatment of diabetes and also advised on the management of the disease by dietary interventions.

Diabetes is a disease which may affect some of clinical enzymes such as alpha amylase , alkaline phosphatase, lactate dehydrogenase, aspartate aminotransferase, lipoprotein lipase.

Rationale:

Glanil is a hypoglycemic drug which is used in the treatment of diabetes. There is need to study the therapeutics of the drug to discorver if it can ameliorate diabetic complications.

Aim: The aim of this work is to study the effect of glanil on Biochemical parameters of alloxan induced diabetic rats.

Objective:

The main objectives of this project are to study:

1 The effect of diabetes on the activities of alkaline phosphatase, alpha amylase activities, concentrations of creatinine, sodium, chloride and total protein.
2 The involvement of these parameters in the diagnosis of diabetes mellitus.
3 To study the effect of diabetes on kidney damage (nephropathy) .
4 To ascertain to what extent glanil can be of help in alleviating the symptoms of diabetes

2.0 CHAPTER TWO

2.1 LITERATURE REVIEW

2.1.1 Diabetes Mellitus

Diabetes mellitus is a lifelong disease and the commonest endocrine disease, which are multi-system disorders resulting from deficiency in the secretion or action of pancreatic hormone insulin and relative glucagons predominance (Femandez and Conget 1984). This in turn produces profound abnormalities of metabolism (Femandez and Conget 1984).

2.1 Classification of Diabetes Mellitus

Diabetes mellitus is classified into four types: type 1, type 2, gestational diabetes and “other specific types” (National Diabetes Data Group 1995)

2.2.1 Types of Diabetes Mellitus

Types 1 diabetes mellitus (formerly called type i, insulin dependent diabetes mellitus (IDDM or juvenile diabetes) is characterized by beta cell destruction caused by an autoimmune process, usually leading to absolute insulin deficiency (McCance *et al* 1997). The onset is usually acute, developing over a period of a few days to weeks. This mainly occurs before the age of 25 (McCance *et al* 1997). Most of the patients have the “immune mediated form” of type I diabetes mellitus with islet cell antibodies and often have other autoimmune

disorders, such as Hashimoto's thyroiditis, Addison's disease, Vitiligo or pernicious anemia (Granberg and Sundkvist 2005).

2.2.2. Type 2 Diabetes Mellitus

Types 2 diabetes mellitus (formerly called non- insulin dependent diabetes mellitus (NIDDM), type 11 or adult- onset) is characterized by insulin resistance in peripheral tissue and an insulin secretory defeat of the beta cell. This is the most common form of diabetes mellitus and is highly associated with a family history of diabetes, older age, obesity and lack of exercise (chang *et al* 2003). It is more common in women, especially women with a history of gestational diabetes. Insulin resistance and hyperinsulinemia eventually lead to impaired glucose tolerance and hyperglycemia (Harris and Flagal 1998).

2.2.3 Gestational Diabetes

Gestational diabetes is diabetes developed during gestation. Most women with gestational diabetes mellitus have normal glucose homeostasis during the first half of the pregnancy and develop a relative insulin deficiency during the last half of the pregnancy leading to hyperglycemia (Lawrence and Sacks 2008). In most women, the hyperglycemia is resolved after delivery but places them at increased risk of developing type 2 diabetes mellitus later in life.

2.2.4 Other Specific Types

Some cases of diabetes are caused by the body's tissue receptors not responding to insulin. Genetic mutations (autosomal or mitochondrial) can lead to defects in beta cell function (Barrett 2001). Any disease that causes extensive damage to the pancreas or associated with excessive secretion of insulin- antagonistic hormone can cause diabetes.

2.3 Signs And Symptoms Of Diabetes Mellitus

The clinical symptoms are polyuria (Frequent urination) and polydipsia (increased thirst) and consequently increased fluid intake. Type 1 diabetes can also cause rapid weight loss. When the glucose concentration in the blood is raised beyond its renal threshold, reabsorption of glucose in the proximal renal tubule is incomplete, and part of the glucose remains in the urine (glucosuria). This increases the osmotic pressure of the urine and inhibits reabsorption of water by the kidney, resulting in increased urine production and increased fluid loss.

Prolonged high blood glucose causes glucose absorption, which leads in changes in the shape of the lenses of the eyes , resulting in vision changes, sustained glucose control usually returns the lens to its original shape (Duncan 2008)

Patients may also have diabetes ketoacidosis (DKA), an extreme state of metabolic dysregulation characterized by the smell of acetone on the patients breath, a rapid, deep breathing, nausea, vomiting and abdominal pain. In severe diabetes

ketoacidosis (DKA), coma may follow, progressing to death (Nosadini and Morocutti 1989).

2.4 Diagnosis of Diabetes Mellitus).

A normal fasting plasma glucose level is less than 110mg per dL(6.1 mmol per L) and normal 2 hour postprandial plasma glucose (2h PPG) level are less than 140mg per dL(7.75 m mol per L) (Santaguida and Hunt 2008). Fasting plasma glucose levels ranging from 110 to 126mg per dL (6.1 to 7.0 mmol per L) is impaired fasting glucose. Levels between 140mg per dL (7.75 mmol per L) and 200mg per dL (11.1 m mol per L) are impaired glucose tolerance. Lifestyle changes, such as weight loss and exercise can reverse it (Harris and Flagal 1998)

2.5 Prevention of Diabetes

Type 1 diabetes risk, is known to depend upon a genetic predisposition based on HLA genotypes and an uncontrolled autoimmune responses that attacks the insulin producing beta cells (Daneman 2006).

Breastfeeding decreases the risk in later life (Naim and Raanan 2001). Giving children 20001µ of vitamin D during their first year of life is associated with reduced risk of type 1 diabetes (Hypponen *et al* 2001).

Types 2 diabetes risk can be reduced by changes in diet (moderate fat intake) and increasing physical activity (Lindstrom *et al* 2006).Prophylactic use of metformin (Knowler *et al* 2002),

rosiglitazone (Gerstein and Holman 2006) or valsartan (Kjeldsen and Julius 2006) delay pregression of diabetes

2.6 Maintenance and Treatment of Diabetes Mellitus

The ultimate goal of the long term management process is prevention of acute and chronic complication of the disease through glycemic control. There is need for patient education, diabetes support, sensible exercise, and self monitoring of blood glucose (Nathan *et al* 2005).

2.6.1Treatment Of Type 1 Diabetes Mellitus

Near normal glycemia in most patients with type1 diabetes is maintained with insulin

Pharmacology of Insulin

Short-Acting Insulin: Regular Insulin

Regular insulin has a peak effect of two to four hours after injection and duration of action ranging from six to eight hours. To ensure insulin availability during food consumption, regular insulin needs to be administered 20 to 30 minutes before meal (Dimitriads and Gerich 1983).

Rapid-Acting Insulin: Insulin Lispro.

Insulin lispro is equivalent to regular human insulin in terms of its binding to the insulin receptor and its effect on cellular glucose uptake (Howey *et al* 1994). The dose of insulin lispro should be adjusted downwards when a low-carbohydrate, high-fat meal is

eaten to prevent hypoglycemia after a meal (Burge and Castillo 1997).

Intermediate-Acting Insulin: NPH AND LENTE INSULIN

NPH insulin shows a more rapid onset and peak of action. NPH insulin is administered at bedtime instead of with evening meal to avoid fasting hyperglycemia (Francis *et al* 1983). Lente insulin has a longer duration of action and a later peak of action than NPH insulin.

Long-Acting Insulin: ULTRALENTE INSULIN.

The duration of action for ultralente insulin ranges from 20 to 24 hours (Seigler *et al* 1991). Ultralente insulin is best administered once daily, it should be given at bedtime (Hirsch 1998).

Inhaled Insulin:

Inhaled insulin is comparable to short-acting subcutaneous insulin with regard to efficacy and hypoglycemic risk. Adverse effect of inhaled insulin include dry cough, a drop in pulmonary function, which is reversible if treatment is discontinued.

Exubera was thus far the only inhaled insulin used in USA and Europe in adults with type 1 and Type 2 diabetes, but was recently withdrawn from the market (Quattrin and Belaarge 2004). The discovery of therapeutic insulin in the early 1920s (Banting *et al* 2007) and its subsequent worldwide distribution has transformed type 1 diabetes from an acutely debilitating disease leading to a certain (sub) acute death into a chronic condition, yet at high risk of

vascular complications and premature cardiovascular death (stamler *et al* 1993).

Insulin is used by about two-third of the body's cells to absorb glucose from the blood for use as fuel or for storage. Insulin is the control signal for conversion of glucose to glycogen for storage in liver and muscle cells. High insulin levels increase some anabolic processes such as cell growth and multiplication, protein synthesis and fat storage (Wood *et al* 1998).

2.6.2Treatment of Type 2 Diabetes Mellitus.

Both non-pharmacologic and pharmacologic therapies are used to reduce insulin resistance in type 2 diabetes. Non-pharmacological approaches include low calorie diet, weight loss and regular vigorous exercise. Pharmacologic approaches included the use of drugs.

SULFONYLUREAS:

Sulfonylurea (tolbutamide) is best used in patients with type 2 diabetes before the age of 40 and duration of disease less than five years before initiation of drug therapy and a fasting blood glucose level less than 300mg per dL (16.7m mol per L)(Mooradian 2006)

METFORMIN

Metformin is a biguanide agent that lowers blood glucose by decreasing glucose output and reducing insulin resistance. Metformin is used as monotherapy or in combination with sulfonylureas for management of type 2 diabetes.

ALPHA-GLYCOSIDASE INHIBITORS:

Alpha-glycosidase inhibitors such as acarbose and miglitol, inhibit the breakdown of complex carbohydrates and delay the absorption of monosaccharide from the gastrointestinal tract (Campbell and White 1996).

TROGLITAZONE.

Troglitazone (thiazolidinediones) is an “insulin sensitizer” that promotes skeletal muscles glucose uptake (Sparano and Seaton 1998). Troglitazone is also effective when used in combination with other oral agents, thereby potentially delaying the need to start insulin therapy (Inzucchi *et al* 1998).

REPAGLINIDE

Repaglinide (prandin) is a non-sulpfonylurea drug. It is suitable for patient with severe sulfa allergy.

GILBENCLAMIDE (GLANIL).

This is a potent ,second generation oral sulfonylurea anti-diabetic agent used as an adjunct to diet to lower blood glucose levels in patients with diabetes mellitus type 2. The hypoglycemic action of glanil is due to stimulation of pancreatic islet cells, which results in an increase in insulin secretion (Mooradian 2006).

Sulfonylurea are believed to bind to ATP-sensitive potassium – channel receptors on the pancreatic cell surface, thereby reducing potassium conductance and causing depolarization of the membrane. Depolarisation stimulates calcium ion influx through voltage-sensitive calcium channels, raising intracellular

concentrations of calcium ions which induces the secretion or exocytosis of insulin (Maran and Gribble 2006). The drug is not effective in the absence of functioning beta –cells as occurs in diabetes mellitus type 1 or when the number of viable beta –cells are low, as occurs in several cases of diabetes mellitus type 2.

Prolonged administration of glanil also produces extra- pancreatic effects that contribute to its hypoglycemic activity. These effects include reduction of basal hepatic glucose production and an enhanced peripheral sensitivity to insulin secondary to an increase in insulin receptors or to changes in the events that follow insulin receptor binding. (Mooradian 2006).

Glanil is rapidly and completely absorbed from the gastrointestinal tract. The on set of action occurs within 2 hours with a maximal decrease in serum glucose occurring within 3 to 4 hours. Glanil is metabolized completely in the liver to two metabolites which are only weakly active. Both unchanged drug and metabolites are excreted equally in the urine and faeces (Mori and Kahn 2007). It can cause elevation of liver enzyme levels and impairment of liver function.

2.7 MEDICINAL HERBS

There are plants which contain substances that can be used for therapeutic purposes or serve as precursor for the synthesis of drugs. The use of medicinal herbs dates back to the history of origin of man (Berson and King 1983). Herbs have been extensively used by man for various curative purposes in different ailments.

Plants contain different active components which are effective in the treatment of various disease and a large percentage of the world population use herbs as medicine (Trease and Evans 1983). Aqueous extract of Bridelia ferruginea leaves have been shown to posses' hypoglycemic effect in diabetic patients as well as alloxan induced diabetes rats (Gill 1992)

Garlic core (Allium sativa) and raw onions (Allium cepa have been used as supplement in diets for the treatment of diabetes in middle East, Europe and Asia (Sheela and Kumura 1995)

Other herbs include Carica papaya, leaf balls of Azadiracta indica (Gill 1992). Hintonia latiflora (Winkelman and Micheal 1986), fruit juice of Monordica charantia (Welihinda and Karunanayake 1986), Magnifera indica, Diascorea Cayenesis

2.8 CURE FOR DIABETES MELITUS

CURE FOR TYPE 1 DIABETES

There is no practical cure. The fact that type 1diabetes is due to the failure of the beta cells in the islets of Langerhans has led to the study of several possible schemes to cure type 1 diabetes by replacing the beta cells of the pancreas (Vinik 2004). Only those type 1 diabetes who have received either a pancreas or a kidney-pancreas transplant (often when they have developed nephropathy and become insulin-independents) may be cured from diabetes

An improved survival rate is improved by pancreas-kidney transplant over a kidney transplant alone (Stratta and Alloway 1998). The

patients generally remain on long-term immunosuppressive drugs to prevent the immune system mounting a host versus graft response against the transplanted organ (Vinik 2004)

COMPLICATAIONS AND PROGNOSIS

Complications of diabetes are far less common and less severe in patients with well-controlled blood sugar levels (Nathan 2005). Smoking, elevated cholesterol levels, obesity, high blood pressure, and lack of regular exercise can accelerate the deleterious effect of diabetes (Cowie and Flegal 1998). Glucose tolerance progressively declines with age, leading to a high prevalence of type2 diabetes and post challenge hyperglycemia in the older population (Harris and Flagal 1998)

2.9.1ACUTE COMPLICATIONS

DIABETIC KETOACIDOSIS

Low insulin level causes the liver to turn to fat fuel (that is ketosis). Elevated levels of ketone bodies in the blood decrease the blood pH, leading to diabetic ketoacidosis (DKA). Ketoacidosis can easily become severe enough to cause hypotension, shock, and death (McGarry *et al 1999)*. The patient recovers when prompt care is given.

HYPERGLYCEMIA HYPEROSMOLAR STATE

When blood glucose level is very high (above 300mg/ dL (16mmol / L), water is osmotically drawn out of cells into the blood. There is increased urine glucose concentration which results in loss of water

and increase in blood osmolarity. The body's cells become progressively dehydrated as water is drawn from them and excreted. This leads to electrolyte imbalance (Rich 2006).

HYPOGLYCEMIA

Hypoglycemia or abnormally low blood glucose is an acute complication of severe diabetes treatments. Consciousness can be altered or even lost in extreme cases, leading is coma, seizure, or even brain damage and death. Diabetic hypoglycemia is caused by several factors such as too much or incorrectly timed administration of insulin, or reduced meal especially glucose containing carbohydrates. Diabetic hypoglycemia is treated with drinks or food. Injection of glucagons or an intravenous infusion of dextrose is used in severe cases (Centofani 1995).

RESPIRATORY INFECTIONS

Hyperglycemia both reduces the function of immune cells and increases inflammation. The vascular effects of diabetes alter lung function which leads to increased in susceptibility to respiratory infections such as pneumonia and influenza (Ahmed *et al* 2008).

2.9.2 CHRONIC COMPLICATIONS.

VASCULAR DISEASE

Chronic elevation of blood glucose level leads to damage of blood vessels (angiopathy). The endothelial cells lining the blood vessels take in more glucose than normal. Since they don't depend on insulin. They then form more surface glycoprotein than normal, and cause the basement membrane to grow thicker and weaker.

Angiopathy is grouped under “micro vascular disease ” (damage to small blood vessels) and “macro vascular disease” damage to the arteries (Monti *et al* 2007).

MICROANGIOPATHY:

Microangiopathy (damage to small blood vessels) can cause any of diabetes retinopathy, diabetes neuropathy, diabetes nephropathy, and diabetes cardiomyopathy (Monti *et al* 2007) .

DIABETIC RETINOPATHY

Diabetes can affect sight by causing cataracts, glaucoma, and most importantly damages to blood vessels inside the eye, a condition known as diabetes retinopathy. When blood vessels in the retina are damaged, they may leak blood and grow fragile.

Symptoms of diabetic retinopathy include difficulty in reading, blurred vision, sudden loss of vision in one eye, seeing rings around lights, dark spots of flashing lights (Ajello 2005). Fortunately, with regular pro eye care and treatment when necessary, the incidence of severe vision loss has been greatly reduced (Fong 2004)

Treatment of retinopathy includes Laser surgery (laser light focused on the damaged retina to seal leaking retina vessels). Also treatment by cryotherapy (freezing) and vitrectomy (removal of blood filled vitreous and replacing with a clear solution (Colucciello 2004)

DIABETIC NEUROPATHY

Diabetic neuropathies are neuropathic disorders resulting from diabetic micro- vascular injury. People with diabetes can develop nerve damage throughout the body. Sometimes, nerve damage is asymptomatic while in some other cases cause pain, tingling or

numbness (loss of feeling) in the hands, arms, feet and legs. Nerve damage can occur in every organ or system including the digestive tract, heart, and sex organs (Boulton *et al* 2005)

CARDIOVASCULAR AUTONOMIC NEUROPATHY

Clinical manifestations of cardiovascular autonomic neuropathy include exercise intolerance, intraoperative cardiovascular lability, orthostatic hypotension, asymptomatic ischemia, painless myocardial infarction and increased risk of mortality (Vinik 2003)

1. EXERCISE INTOLERANCE

In diabetic individuals with cardiovascular automatic neuropathy, exercise tolerance is limited as a result of impaired parasympathetic responses that would normally enhance cardiac output and result in directional peripheral blood flow to skeletal muscle (Vinik 2003)

2 INTRAOPERATIVE CARDIOVASCULAR LABILITY

There is a 2 - to – 3 fold increase in cardiovascular morbidity and mortality intraoperatively for patients with diabetes (Ziegler 1999). Patients with severe autonomic dysfunction have a high risk of blood pressure instability (Latson *et al* 1994). Intraoperative hypothermia causes decrease in drug metabolism and affect wound healing, and impaired hypoxic-induced ventilatory drive (Sobatka *et al* 1986)

3 ORTHOSTATIC HYPOTENSION:

This is a fall in blood pressure that is > 20-30 mm Hg for systolic or >10 mm Hg for diastolic in response to postural change from supine to standing (Purewal and Watkins 1995). A change from lying to standing normally results in activation of a baroreceptor -initiated,

centrally mediated sympathetic reflex, resulting in an increase in peripheral vascular resistance and cardiac acceleration (Ziegler 1999). Orthostatic hypotension is characterized by a defect in this reflex arc, resulting in signs such as weakness, dizziness, visual impairment

4 PAINLESS MYOCARDIAL ISCHEMIA

Impaired afferent signaling resulting from autonomic dysfunction is associated with failed signal transmission from the thalamus to the frontal cortex (Rosen and Camici 2000)

5 INCREASED RISK OF MORTALITY

Impaired autonomic control of heart rate is linked to increased risk of mortality. Reduced parasympathetic function or increased sympathetic activity may provide the propensity for lethal arrhythmias (Maser *et al* 2003)

Treatment of diabetic neuropathy include tight glucose control (Larsen *et al* 2004), photo energy therapy, epalrestat, antioxidants (Tankova *et al* 2004), angiotensin type 1 blockers (Didangelos *et al* 2002), aldosterone blockers (Pitt and Zonnad 1999), calcium-channel blockers (Kailasam *et al* 1995)

DAIBETIC NEPHROPATHY

Diabetic nephropathy is damage to the kidney which can lead to chronic renal failure, eventually requiring dialysis (Bakris *et al* 2000) or kidney transplant

EPIDEMIOLOGY

In 2000, according to the World Health Organization, at least 171 million people worldwide suffer from diabetes or 2.8% of the populations (Wild *et al* 2004). It's incidence is increasing rapidly, and it is estimated that by the year 2030 this number will almost double (Wild *et al* 2004)

In 2008, there were about 24 million people with diabetes in the United States alone, from these, 5.7 million people remain undiagnosed. Other 57 million people are estimated to have pre-diabetes. The American Diabetes Association cite the 2003 assessment of the National Center for Chronic Disease Prevention, and Health Promotion that 1 in 3 Americans born after 2000 will develop diabetes in their lifetime (Narayan and Thompson 2003). The National Health and Nutrition Examination Survey (NHANES 111) demonstrated that in the population over 65 year old, 18% to 20% have diabetes, with 40% having either diabetes or its precursor form of impaired glucose tolerance (Harris and Flagal 1998).

2.11 EFFECT OF ALLOXAN ON THE PANCREATIC ISLET CELLS.

Alloxan reacts with the insulin release mechanism in two stages :first stimulation ,second inhibition of insulin release. A brief burst of insulin release was detected after five minutes exposure to alloxan in isolated islets and isolated perfused rats pancreas (Weaver *et al* 1978)

The second stage is its chemical reaction with the receptor to prevent further stimulation with alloxan or D-glucose. Insulin release by alloxan is like glucose –induced insulin release, dependent upon extra cellular calcium and associated with an increased uptake of calcium. The subsequent inhibition of insulin release results in insulin –dependent type of diabetes mellitus as insulin could not be released by the beta cells of islets of langerhans in the pancreas. This now leads to increased blood glucose level (hyperglycemia), deranged carbohydrate metabolism and at about 7 hours later hypoglycemia with significant increased serum immuno- reactive by insulin. After 24 hours the diabetic state became evident characterized by polyuria, glycosuria, hyperglycemia and a fall in the pancreatic immunoreactive insulin content, increasing urine volume, rapid and irreversible necrosis of the pancrease and finally death of the fasted animal. High dosage of alloxan is also known to inhibit glucagon release for amino acid uptake (Weaver *et al* 1978). Mechanism is not yet known but is assumed that the direct toxic action of alloxan affects both alpha and beta cell types transiently stimulating and then permanently blocking the beta cells and irreversibly inhibiting the alpha cells (Pagliara and Stillings 1977)

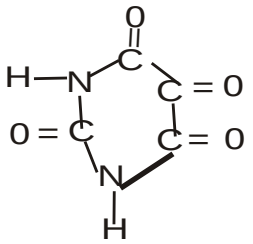


Fig 1: alloxan

2.11

fig 2 . Molecular model of alkaline phosphatase (Stockham and Scott 2002).

ALKALINE PHOSPHATE ACTIVITY

Alkaline phosphatase [E.C.3.1.3.1.ALP] is an orthophosphoric monoester phosphohydrolase. It catalyzes the hydrolysis of a member of phosphtase esters, transferring the phosphate group to an acceptor molecule.

Alkaline phosphatase is a membrane bound metalloenzyme that carry out the enzymic activity in an alkaline environment (pH of 10) (Bain *et al* 2003). Although their exact physiological roles are not recognized, they are typically membrane –bound enzymes that are often associated with brush borders. Increases in membrane permeability does not cause their release into plasma (Stockham and Scott 2002). The isoenzymes of alkaline phosphatase that are of clinical use in medicine include the hepatic, corticosteroid induced, bone, intestinal and placental forms.

CLINICAL IMPORTANCE OF MEASURING ALKALINE PHOSPHATASE ACTIVITY

A serum alkaline phosphatase measurements most useful clinical attribute is its sensitivity in distinguishing hapatobiliary disease (Ettinger and Feldman 2000). However, due to its numerous isonezymes its presence in non-hepatic tissue, and its sensitivity to drug induction has a low specificity for hepatobiliary disease (Ettinger and Feldman 2000). There are no significant causes of decreased alkaline phosphatase (Willard *et al* 1999). Also, there is no correlation between the magnitude of increase in alkaline phosphatase and the progress or seriousness of the disease (Willard *et al*1999). Although mainly used for evaluation of liver disease, there are some other uses of alkaline phosphates measurement.

The measurement of corticosteriod - induced alkaline phosphatase can be used as a screening test for hyperadrenocorticism (Stockham and Scott 2002)

Alkaline phosphatase has been shown to be a prognostic indicator for canine osteosarcoma (Kirpensteijn *et al* 2002). Measurement of alkaline phosphatase concentration in urine has been used as an indicator of early toxic tubular injury (Wisloff and Flayen 2003)

OPTIMAL RANGE OF ALKALINE PHOSPHATASE ACTIVITY

The optimal range of alkaline phosphates depends on the age. A growing adolescent will have a much higher alkaline phosphatase than a full grown adult because the osteoblasts are laying down bone very rapidly. For an adult, 50-75mg/ dL is considered a reasonable optimal range (Li-fern and Rajasoorya 1999). Evaluation of serum alkaline phosphatase concentration in patients with diabetes mellitus has been reported (Maxwell and Hemie 1986)

ALLKALINE PHOSPHATES ISOENZYMES

HEPATIC ISOENZYME

The hepatic isoenzyme of Alkaline phosphatase (L-ALP) is an induced (cholestatic) enzyme (Stockhan and Scott 2002,) that are relatively specific for cholestasis. Cholestasis is decreased secretion of bile from extrahepatic or intrahepatic cuases (Bain *et al* 2003). When considering various types of hepatic diseases, focal or diffuse intrahepatic or extrahepatic cholestasis causes the greatest increases in serum alkaline phosphatase activity (Ettinger and Feldman 2000). When bile concentration in the liver are increased, L-ALP production is triggered and L-ALP gathers on the sinusoidal side of the hepatocyte. This increased sinusoidal enzyme

concentration is the ultimate reason for the measured increase in serum alkaline phosphatase activity (Skockhan and Scott 2002)

CONDITIONS FOR INCREASED HEPATIC ALKALINE PHOSPHATASE (L-ALP).

These include degenerative disease such as hepatic necrosis, metabolic disorders such as lipidosis, diabetes mellitus (Maxwell and Hemie 1986), induction by drugs or hormones such as phenobarbitol, corticosteriods.

CORTICOSTEROID INDUCED ISOENZYME

The corticosteriod induced isoenzyme of alkaline phosphatase is located in the area of the hepatocyte membrane that constitutes the bile canaliculi (Sanecki *et al* 1987)

Both endogenous and exogenous glucocorticoid (including topical and opthalmic medications) can lead to noticeable increases in alkaline phosphatase activity (Bain *et al* 2003).

During corticosteroid treatment, the initial rise in alkaline phosphatase activity is due to the hepatic isoenzyme of alkaline phosphatase (L-ALP).

BONE ISOENZYMES

Lytic or proliferate lesions of bone result in increased osteoblastic activity. The increased activity of bone alkaline phosphatase (B-ALP) adds to the total activity of alkaline phospohatase in serum (Bain *et al* 2003). Active reabsorption of bone also can result in increased B – ALP activity

INTESTINAL AND PLACENTAL ISOENZYMES

The intestinal isoenzyme has a very short half-life. The placental isoenzyme may cause increased serum or plasma alkaline phosphatase activity during late term pregnancy (Bain *et al* 2003)

The intestinal isoenzyme is involved in some metabolite transport across cell membrane (Dufuor 2000)

2.13 CREATININE CONCENTRATION.

Creatinine depend largely on glomerular filtration for their excretion and they are not reabsorbed as readily as the electrolytes (Guyton and Hall 1996) Creatinine is not reabsorbed so that the excretion rate is equal to the rate at which it is filtered. Creatinine filtration rate = GFR x plasma creatinine concentration = creatinine excretion rate.

BIOCHEMISTRY OF CREATININE.

Creatinine is synthesized in the kidney, liver and pancreas by two enzymatically mediated reactions. The first reaction involves transamidation of arginine and glycine form guanidinoacetate. In the second reaction, methylation of guanidinoacetate occurs with S-adenosyl methionine as the methyl donor. Creatine is then transported in the blood to other organs such as muscle and brain where it is phosphorylated to phosphocreatine (a high energy compound). The interconversion of phosphocreatine and creatine is a particular feature of metabolic processes of muscle contraction (Murray 2000). Some of the free creatine in the muscle is spontaneously converted to creatinine, its anhydride. Between 1% and 2% of muscle creatine is converted to creatinine daily, which is

excreted from the kidney. Creatinine is produced at a steady rate and is affected very little by diet or by normal physical activities.

CLINICAL UTILITY: Creatinine is endogenously produced and released into the body fluids at a constant rate and its plasma level maintained within narrow limits, its clearance may be measured as an indicator of GFR (Mitch 1987). However a small quantity of creatinine is reabsorbed by the tubules and a small quantity of creatinine appearing in the urine (7%-10%) is due to tubular secretion.

Increased serum creatinine can be caused by renal failure due to primary renal disease , cardiac failure, salt depletion, shock, diarrhea, vomiting, intestinal obstruction, diabetic coma, Addison's disease.

2.14 ALPHA –AMYLASE ACTIVITY IN DIABETES.

Alpha amylase in the mouth hydrolyzes the internal glucosidic linkages of starch, producing short polysaccharides and oligosaccharides. Pancreatic alpha –amylase secreted into the small intestine catalyses the further break down of polysaccharides into maltose and maltotriose (Erlandsen and Abola 2000)

Insulin stimulates exocrine activity while glucagons has inhibitory role both in the synthesis and secretion of amylase and HCO^-_3 ions (Hardt and Krauss 2000).

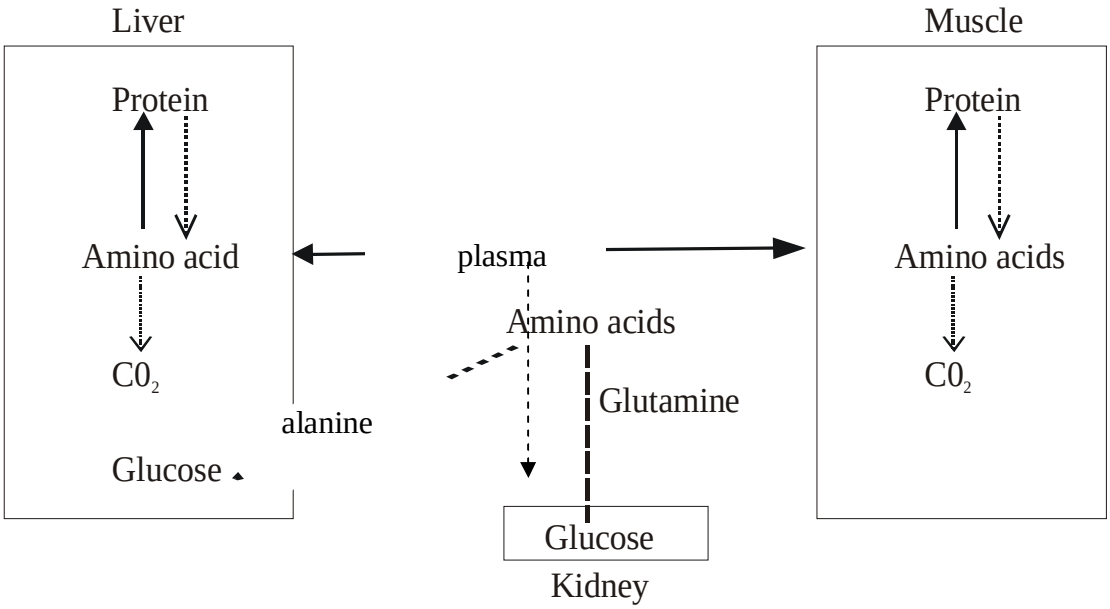
It is observed that serum alpha -amylase activity drops initially due to impaired pancreatic exocrine secretion as a result of decrease in the insulin stimulatory action (Shimuzu and Hayashi 2000). Transcient increase in enzyme activity follows which reflects

the release of the enzyme from cellular compartments caused by increased destructive processes (Maciejewski and Burski 2001). A definite depletion of alpha-amylase in the exocrine cells causes subsequent decrease in enzyme activity (Maciejewski and Burski 2001).

2.15 **PROTEIN CONCENTRATION IN SERUM AND TISSUE**.

Proteins are targets for oxidative stress in diabetes leading to diabetic complications. Hyperglycemia includes the overproduction of oxygen free radicals and consequently increases the protein oxidation. Increased protein oxidation causes reduced plasma total protein. There is profound increases in photolytic products of gyrated and oxidized protein in diabetic patients, concurrent with much lower increases in protein gyration and oxidation adduct residues. (Wolff and Jiang 2009)

There is no proteolysis in kidney during diabetes mellitus.



Joslin diabetic 1994, 7, 122.

Fg 2**:Protein concentration in diabetic tissues**

2.16 SODIUM CONCENTRTION IN DABETES MELLITUS.

Diabetes mellitus is frequently associated with electrolytes and acid-base disturbances.

Sodium is the major cation of extra cellular fluid. It plays a central role in the maintenance of the normal distribution of water and the osmotic pressure in the various fluid compartment (Tietz 1987). Hyponatremia (low serum sodium level), is found in conditions such as severe polyuria, metabolic acidosis, Addison's disease, diarrhea and renal tubular disease (Tietz 1987).

Hypernatremia is the consequence of osmotic diuresis. Loss of water in excess of Na^+ + K^+ will directly raise the plasma sodium concentration unless there is a concomitant increase in fluid intake.

Hypernatremia is common after insulin therapy especially in diabetic coma as both glucose and water re-enter the cells (Arieff 1997)

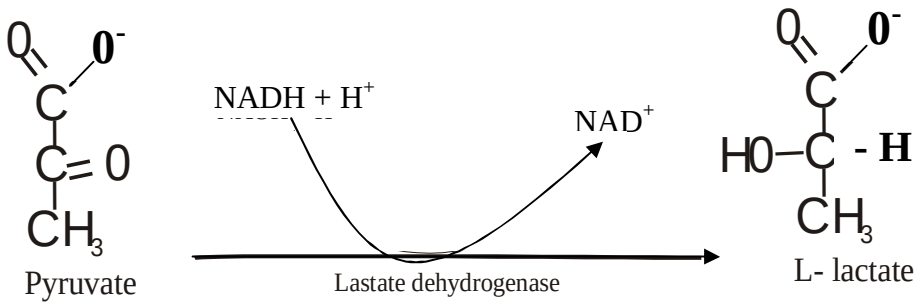
2.17 CHLORIDE CONCENTRATION IN DIABETES MELLITUS

Chloride, a major anion is important in the maintenance of the cation/anion balance between intra-and extra-cellular fluids. This electrolyte is therefore essential to the control of proper hydration, osmotic pressure, and acid/base equilibrium.

Low serum chloride is found with extensive burns, excessive vomiting, intestinal obstruction, nephritis, metabolic acidosis and in Addisonian crisis(Tietz 1987) . Elevated serum chloride value may be seen in dehydration,hyperventilation, congestive heart valve, and prostatic or other types of urinary obstruction (White *et al* 1998).

2.18 SOME ENZYME ACTIVITIES THAT ARE EFFECTED BY DIABETES MELLITUS .

2.18.1 EFFECT OF DIABETES MELLITUS ON PLASMA LACTATE DEHYDROGENASE



Under anaerobic condition, lactate dehydrogenate catalyzes the reduction of pyruvate to L-lactate (Oliver and Flynn 1993)

There are contradiction in the reports about the plasma level of lactate dehydrogenase (LDH). Most research done by Oliver *et al*

(1993) and Tanaka and Nanbara (1988) show that there was no significant increase in the plasma lactate dehydrogenase level between the diabetes and control subjects.

In another research done by Jones and Grant (1988) and Zappacosta and Rossi (1995) indicated that lactate dehydrogenase levels were higher in patients with diabetes mellitus than those in normal subjects. On the other hand (Cai 1989) observed decrease in lactate dehydrogenase in diabetic subjects. Increase in enzyme level may be contributed by: malnutrition, hepatic anoxia and infection in diabetes.

2.18.2 EFFECT OF DIABETES MELLITUS ON PLASMA GLUTAMIC OXALOACETIC TRANSAMINASE (GOT) (ASPARTATE AMINOTRANSFERASE (AST)

There are contradiction in the results of effects of diabetes mellitus on glutamic oxaloacetate transaminase. Fernandez and Conget (1984) reported that the level of GOT did not change in diabetes mellitus compared with normal subjects. Tanaka and Nanbara (1988) indicated that GOT level was higher in diabetes mellitus than those of normal subjects. On the other hand, Awaji *et al* (1990) observed decreases in GOT between diabetes and normal subjects.

These differences can be influenced by the enzyme being released from other organs which may cause changes in levels of enzyme.

2.18.3 EFFECT OF DIABETES MELLITUS ON HUMAN ADIPOSE TISSUE LIPOPROTEIN LIPASE ACTIVITY

Lipase catalyzes the hydrolysis of stored triacylglycerols, releasing fatty acids for export to the site where they are required as fuel (Brunzel 2000). Insulin regulates human adipose tissue lipoprotein lipase activity. Defects in insulin secretion is association with low adipose tissue lipoprotein lipase and may lead to hypertriglyceridemia in diabetes mellitus (Nilsson 1999).

2.18.4 EFFECT OF DIABETES MELLITUS ON SERUM/PLASMA GAMMA- GLUTAMYL TRANSFERASE ACTIVITY.

There is correlations between gamma glutamyltransferase activity and body mass index, serum lipids, lipoproteins, glucose, insulin and blood pressure (Whitfield and Zhu 2002).

Serum gamma glutamyltransferase is a predictor for developing metabolic syndrome and type 2 diabetes mellitus (Nakanishi and Suzuki 2004)

2.18.5 EFFECT OF DIABETES MELLITUS OF PLASMA ALANINE AMINOTRANSFERASE (ALT) (GLUTAMATE-PYRUVATE TRANSAMINASE (GPT)

Alanine aminotransferase catalyzes the transfer of α-amino group of glutamate to pyruvate. The alanine formed passes into the blood to the liver. In the hepatocytes, alanine aminotransferase catalyzes the transfer of amino group from alanine to α-ketoglutarate, forming pyruvate and glutamate (Coomes 1997).

Serum alanine aminotransferase was significantly associated with metabolic syndrome in men but not in women (Kim and Chio 2005)

2.18.6 EFFECT OF DIABETES MELLITUS ON NA^+K^+ ATPase ACTIVITY

Diabetes mellitus (both type 1 and 2) induces a reduction in erythrocyte membrane Na^+K^+ATPase activity, which results in homodynamic dysfunction due to altered microvascular blood flow, and raised fluidity and complications such as nephropathy, neuropathy, cardiovascular disorders and microangiopathy (Kunt *et al*1999).

Decreased Na^+K^+ATPase in diabetes mellitus is associated with microalbuminuria, hyperkalaemia and elevated serum sialic acid (Mimura and Makino 1994). Glucose in diabetic state exhibited direct toxicity on erythrocyte membrane protein including Na^+K^+ATPase through non-enzymatic glucosylation (Nandhini and Anuradha2003)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 ANIMALS

Fifteen albino male rats (*Sprague dawley* strain) of weights ranging from 145 g to 175 g were obtained from university of Nigeria Nsukka.

3.1.2 FEED

The rats were fed on rat palletized feed obtained from Grand cereal and oil mills ltd, Bukuru, Jos.

3.1.3 CHEMICALS

Some of the chemicals include: Chloroform (BDH lab ltd, Poole England). Anhydrous sodium carbonate (May and Baker, Dagenham, England).

Sodium hydrogen carbonate (May and Baker Dagenham, England)

Magnesium sulphate (E. Merck Darmstadt W. Germany)

Para-nitrophenyl phosphate (BDH lab ltd. Poole, England)

Sodium hydroxide (BDH lab ltd, Poole England)

Alloxan (BDH lab ltd, Poole England)

Sodium chloride (BDH lab ltd, Poole England)

Amylase kit (Teco diagnostic, California USA)

Creatinine kit Randox lab ltd, United Kingdom).

Chloride kit (Teco diagnostic, California USA)

Protein kit (Teco diagnostic California, USA)

Sodium kit (Teco diagnostic California USA).

Glanil (Gilbenclamide Nigeria

3.1.4 APPARATUS.

Some of the apparatus used includes:

- Electric centrifuge (B. Bain scientific and instrument company, England)
- Gallenkamp magnetic stirrer thermostat (B.Bain scientific and instrument company, England).
- Thermometer (E.C. Apparatus Corporation St Petersburg, Florida).
- Adams weighing balance (B. Bain scientific and instrument company, England)
- Potter Elvejheim homogenizer with Teflon
- Pestle (E. C. Apparatus Corporation, St Petersburg, Florida).
- Glucometer (Roche diagnostics GMBH, Germany)
- Ultrospec 3100 pro (Amersham Biosciences, England)
- Micropippette (B. Bain scientific and instrument company, England)

3.2.1 INDUCTION OF DIABETES MELLITUS IN RATS

The rats were placed on the same pellet feed for two weeks and the initial weights were measured and recorded.

The rats were fasted for 12 hours as suggested by Kass and Waisbren (1945), and the initials blood glucose concentration measured using glucometer and grouped into three. Stock solution of alloxan (5%) was prepared by dissolving 5 g of alloxan in 100 ml of distilled water. This solution equivalent to 100 mg/kg body weight was injected into the rats through intraperitoneal route. The rats were

placed on the pellet feed for eight days. Diabetes was established by monitoring the blood glucose using glucometer.

3.2.2 **BLOOD ANALYSIS**.

After fasting the rats for 12 hours, (overnight) their fasting blood sugar level was measured and recorded. Picric acid was used to mark and identify individual rats. The test groups were administered with alloxan to induce diabetes mellitus after which they received glucose prepared by dissolving 75 g of glucose in 1 litre of distilled water. The control rats received distilled water.

The blood glucose was monitored at interval. The tails of the rats were cleaned with absolute alcohol before and after bleeding to avoid contamination and vaseline were used to warm up the tail to allow blood flow.

3.2.3 TREATMENT OF DIABETES

Having established diabetes on the sixth day, the group to be treated was administered with glanil, using 1 ml syringe. 10 mg of glanil was dissolved in 50ml of distilled water and 0.2 ml was administered orally.

3.2.4 COLLECTION OF TISSUE

The rats were placed under an aesthetic (chloroform) on the eight day and blood drawn by cardiac puncture. The rats were sacrificed and dissected to collect kidney and liver. The tissues were kept in ice bath. The blood was centrifuged to obtain serum while the tissue was weighed.

3.2.5 PREPARATION OF TISSUE HOMOGENATE

One gram each of kidney and liver were homogenized using 8 ml of saline (0.9% NaCl). The homogenate was centrifuged at 3000 rpm (resolution per minute) for 10 minutes and the supernatant collected and preserved in the deep freezer temperature according to (Marsh and Drabkin 1991).

3.2.6 PREPARATION OF SERUM

The serum was prepared according to the method of Robinson and Robert (1961). The blood was drawn from the rats by cardiac puncture using 5 ml syringe into different centrifuge tubes containing no anticoagulant. They were incubated at room temperature (25^0C)for about 30 – 45 min and centrifuged for 15 min at 3000 g. The clear supernatant (serum) was aspirated carefully at room temperature into tubes with covers and stored in the freezer (-5^0C).

3.3.0 ASSAY PROCEDURE.

3.3.1 ASSAY FOR TISSUE AND URINE ALKALINE PHOSPHATASE ACTIVITY (WRIGHT *et al* 1972)

PRINCIPLE

Alkaline phosphatase catalyses the hydrolysis of para-nitrophenyl phosphate to para-nitrophenol, which is yellow in colour in alkaline solution. Addition of NaOH terminates the enzyme activity and brings out the colour of para-nitrophenol.

P-nitrophenyl +H_2O Alkaline phosphatase P -nitrophenol

Phosphate ⟶ + phosphate

The method of assay used was that of Bessey et al (1946) and modified by Wright *et al* (1972) in which the hydrolysis of para-nitrophenyl phosphate was monitored spectrophotometrically.

Since the hydrolysis product, para-nitrophenol, shows a characteristic yellow colour in alkaline solution, the reaction was stopped by addition of IN sodium hydroxide and the extinction read at 400nm. The initial incubation of enzyme with buffer serves to inactivate microsomal phosphatase (Shibko and Tappel 1965) .

PREPARATION OF REAGENTS

Para-nitrophenyl phosphate (19mM): Para-nitrophenyl phosphate $NO_2C_6H_4$ Na_2PO_4. $6H_2O$ =371.15MW (1.76 g) was dissolved in distilled water and made up to 250ml

Sodium hydroxide NaOH (1N). NaOH (40 g) was dissolved in distilled water and made up to 1 litre

Carbonate bicarbonate buffer (O.I M) (pH 10.1)

Stock solution:

Solution of anhydrous sodium carbonate (0.2 M) was made by dissolving 21.2 g of Na_2CO_3 in distilled water and made up to 1000 ml.

Solution of sodium bicarbonate (0.2 M) was prepared by dissolving 16.8 g of $NaHCO_3$ in distilled water and made up to 1000 ml.

Mixtures of 30.0 ml of anhydrous sodium carbonate solution and 20.0 ml of sodium bicarbonate solution and made it up to 250 ml with distilled water gives pH 10.1.

Magnesium sulphate (0.1M): $MgSO_4$ (12.0 g)was dissolved in 1000 ml of distilled water.

PROCEDURE

The assay mixture is made up as follows:

	Blank (ml)	Test (ml)
Na_2C0_3/ $NaHC0_3$ buffer 0.1M (Ph 10.1)	2.2	2.2
Mg $S0_4$ (O.1M)	0.1	0.1
Para-nitrophenyl phosphate (19 mM). Freshly prepared Mixture is equilibrated at 37^0C in water bath for 10 minutes	0.5	0.5
Aliquot (Enzyme source)	-	0.2
Distilled water	0.2	-
Shake mixture thoroughly and incubate for 10 minutes at 37^0C		
Stop reaction with IN NaOH	2.0	2.0

Extinction (Absorbance) was taken at 400 nm

CALCULATION

The method of calculation used was that of (Ngaha 1974). One unit of alkaline phosphatase activity is defined as an increase in extinction of 0.001 /ml of enzyme solution/ minute. The extinction coefficient of 1 µmole of para-nitrophenol in an alkaline solution of 1 ml volume and 1 cm light path is 18.8 at 400 nm. The change in extinction / minute (ΔE/min) was therefore converted to enzyme activity in international units (nM/Min/ml)

$$\text{Activity (nM / min/ml)} = \frac{\Delta E \times 1000 \times V \times f}{18.8 \times v \times d} \quad \text{---(a)}$$

Where

ΔE = Change in extinction of the reaction mixture per minute.

v = Volume of enzyme source sample

f = Dilution factor

1000 = this factor is introduced so that the enzyme activity can be expressed in µmole /min/L or nM/ min/ ml

V = Final volume of reaction mixture

d = the light path length (1cm)

i) for tissue

if the activity from (a) above = X

$$\text{The specific activity} = \frac{X(\text{nM /min /ml}}{\text{Protein conc (mg /ml)}} \quad \text{---- (b)}$$

= DnM /min mg protein

3.3.2 DETERMINATION OF SERUM AND TISSUE AMYLASE USING KIT (MODIFICATION OF WALLENFELS *et al* 1978).

The determination of amylase activity in serum and urine is commonly performed for the diagnosis of acute pancreatitis. In acute pancreatitis, amylase concentration are elevated for longer periods of time in urine than in serum(Wallenfels 1978).

PRINCIPLE:

P – nitrophenyl –D- Maltoheptaoside (PNPG7) is the substrate with the terminal glucose blocked to reduce spontaneous degradation of the substrate by glucosidase and glucoamylase.

Amylase hydrolyzes ρ – nitrophenyl –D- maltoheptaoside (PNPG7) to ρ–nitrophenyl maltotiose (PNPG3) and maltotetraose. Glucoamylase hydrolyses PNPG3 to ρ- nitrophenylglucoside (PNPG1) and glucose. Then PNPG1 is hydrolysed by glucosidase to glucose and ρ – nitrophenol, which produces a yellow colour. The rate of increase in absorbance is measured at 405 nm and is proportional to the amylase activity in the sample.

PNPG7 $\xrightarrow{\text{amylase}}$ PNPG3 + Maltotetraose

PNPG3 $\xrightarrow{\text{Glucoamylase}}$ PNPG1 +Glucose.

PNPG1 $\xrightarrow{\text{Glucosidase}}$ ρ –nitrophenol + Glucose.

PROCEDURE

One milliliter of reagent was pipetted into test tubes, pre- warmed at 37^0C for 3 minutes. The sample (0.025 ml) was added and absorbance read after 15 seconds at 405 nm using distilled water as blank.

Calculations

$$\frac{\Delta Abs/min \times T.V \times 1000}{M.M.A \times S.V \times LP} = \text{1U/L amylase in sample}$$

Where

ΔAbs/min = Absorbance difference per minute

T.V. = Total assay volume (1.025 ml)

1000 = Conversion 1U/ml to IU/L

M.M.A = Millimeter absorptivity of p-nitrophenol (8.5)

S.V = Sample volume (0.025 ml)

L.P = Light path (1cm)

3.3.3 ESTIMATION OF CREATININE IN SERUM AND TISSUE USING KIT BY (JAFFE METHOD 1976)

PRINCIPLE

Creatinine in alkaline medium reacts with picrate to form a coloured complex. The rate of formation of the complex is measured.

PROCEDURE:

Reagent mixture (2.0 ml) was pipetted into tubes labeled blank, standard, and sample. Standard solution 1 (0.2 ml) was pipetted into the standard tube, 0.2 ml of sample was pipetted into the respective sample tubes and 0.2 ml of distilled water pipetted into the blank. Absorbance A_1 of the standard and sample were read, two minutes later absorbance A_2 of standard and sample were read at 510 nm

Calculations

$A_2 - A_1$ = A sample or A standard

Concentration of creatinine in serum or tissue

$\frac{\text{A sample}}{\text{A standard}} \times 2$ = mg/dl.

$\frac{\text{A sample}}{\text{A standard}} \times 177 = \mu mol/l$

3.3.3 ESTIMATION OF TISSUE AND SERUM TOTAL PROTEIN USING KIT.

PRINCIPLE

Cupric ions, in an alkaline medium interact with protein peptide bonds resulting in the formation of a coloured complex.

PROCEDURE

Samples (0.02 ml)was pipetted in the respective tubes and 0.02 ml of standard was pipetted in the standard tube. Then 0.02 ml of distilled water was pipetted into the blank. One milliliter of reagent was pipetted into all the tubes and incubated for 30 minutes at 20^0C to 25^0C. Blank was used to zero the spectrophotometer, the absorbance of standard and samples were read at 540 nm.

Calculations

Total protein conc. (g/l) = 190 x A sample

Total protein conc. (g/dl) = 19 x A sample

When using a standard

$$\text{Total protein conc.} = \frac{\text{A sample}}{\text{A standard}} \times \text{standard conc.}$$

3.3.5 ESTIMATION OF TISSUE SODIUM USING SODIUM KIT

(Modification of Maruna's method (1968)

PRINCIPLE:

Sodium is precipitated as a triple salt, sodium magnesium uranyl acetate, with the excess uranium then reacting with ferrocyanide, producing a chromosphores whose absorbance varies inversely as the concentration of sodium in the test specimen.

PROCEDURE

Filtrate reagent (1.0 ml) was pipetted to all tubes, 0.05 ml of sample was pipetted to the test tubes and 0.05 ml of distilled water to the blank. The tubes were shake vigorously for 3 minutes and centrifuged at 3000 rpm for 10minutes and supernatant collected.

Acid reagent (1.0 ml) was pipetted into the tubes and 0.05 ml of supernatant pipetted into respective tubes and mixed. Colour reagent (0.05 ml) was pipetted into all the tubes and mixed. Absorbances of all the tubes were read using distilled water blank to zero spectrophotometer at 550 nm.

Calculations

$$\frac{\text{Abs of blank} - \text{Abs of S}}{\text{Abs of blank} - \text{Abs STD}} \times \text{Conc. of std (mEq/L)} = \text{Conc. of S(mEq/L)}$$

Abs = Absorbance

S = Sample

STD = Standard

Conc of STD = 150 mEq/L

3.3.6 ESTIMATION OF TISSUE CHLORIDE USING KIT (colorimetric method of Skeggs and Hochestrasser 1976)

PRINCIPLE

$Hg(SCN)_2 + 2Cl^- \longrightarrow HgCl_2 + 2SCN^-$

$3SCN^- + Fe^{3+} \longrightarrow 4Fe(SCN)_3$ red complex

Chloride ions form a soluble, non ionized compound, with mercuric ions and will displace thiocyanate ions from non-ionized mercuric thiocyanate. The released thiocyanate ions react with ferric ions to form a colour complex that absorbs at 480 nm. The intensity of the colour produced is directly proportional to the chloride concentration.

PROCEDURE

Chloride reagent (1.5 ml) was pipetted into tubes. Calibrator (0.01ml) was pipetted into the calibrator tube and 0.01 ml of samples were pipetted into respective tubes and 0.01 ml of distilled water into the blank tube. The tubes were allowed to stand for five minutes and absorbance read at 480 nm using blank to zero the spectrophotometer.

CALCULATIONS:

$$\frac{\text{Absorbance of unknown}}{\text{Absorbance of calibrator}} \times \text{conc. of calibrator} = \text{conc of chloride (mEq/L)}$$

Concentration of calibrator = 100 mEq/L

CHAPTER FOUR

Results:

Mean blood glucose concentrations of albino rats before and after alloxan and glanil administration (mg/100 ml).

Parameter	Day	Control rats	Alloxan infused rats	Glanil treated rats
Blood glucose mg/100ml.	zero	100.20±3.06	95.60±9.52	100.80±4.99
	2	100.20±3.06	60.80±16.94	73.60±28.60
	6.	100.20±3.06	262.80±41.78	245.60±39.94
	8.	100.20±3.06	291.00±17.04	88.25±10.80

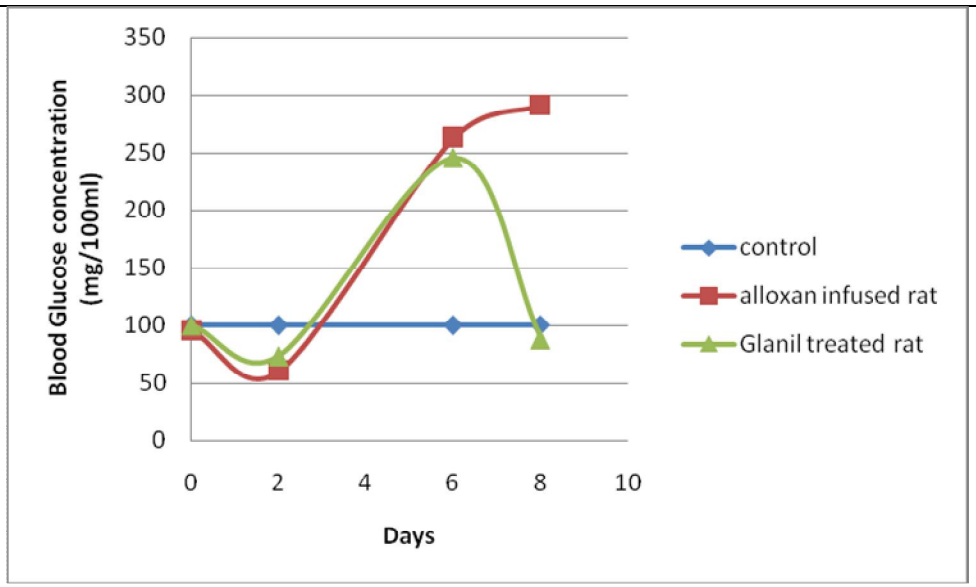


Fig 3: Blood Glucose Concentration (mg/100ml)

Values are expressed in mean ± standard deviation for n=5. There was no significant difference ($p>0.05$) in the serum glucose concentration of the control and the test subjects before alloxan administration. Hyperglycemia was significantly higher after alloxan treatment (on the eight day) at ($p<0.05$) in the diabetic subject, compared to the control rats. After treatment of the rats with glanil, there was a return to normal fasting blood glucose level. The body weights of the rats did not change significantly after the study.

TABLE 2

Mean serum, kidney and liver alkaline phosphatase activity at ΔA 400nm in μm/min/ml for n=5.

Parameter	Control rats	Alloxan infused rats	Glanil treated rats
Serum ALP (μm/min/ml)	3.12±0.44	6.50±0.50	8.17±2.10
Kidney ALP (μm/min/mg/protein)	5.52±2.67	8.72±0.99	3.44±0.24
Liver ALP (μm/min/mg/protein)	3.30±2.37	8.49±3.76	4.03±2.75

Values are expressed in mean ± standard deviation for n=5. There was a significant increase at $p<0.05$ in serum, kidney, and liver alkaline phosphatase activity of diabetic rats compared to the control

subjects after eight days. There was a significant increase at $p<0.05$ in serum alkaline phosphatase of the treated rats when compared to control rats.

TABLE 3

Mean serum, kidney, liver amylase activity at ΔA 405 nm is IU/L for n=5.

Parameter	Control rats	Alloxan infused rats	Glanil treated rats
serum amylase IU/L	60.49±19.23	140.78±8.15	80.87± 23.34
Kidney amylase IU/L	48.92±25.82	69.80±21 .48	92.70±25.00
Liver amylase IU/L	63.40±46.22	35.38±14.60	73.45±17.06

There was a significant increase in serum amylase activity of the diabetic group when compared with the control and treated rats at $p<0.05$.

TABLE 4

Mean serum, kidney and liver creatinine concentration at 510 nm in µmol/L for n= 5

Parameter	Control rats	Alloxan infused rats	Glanil treated rats
Serum creatinine µmol/L	472.28±69.13	619.48±76.41	203.23±57.72
Kidney creatinine µmol/L	69.23±18.25	91.20±13.23	94.35±41.38
Liver creatinine µmol/L	280.06±68.63	687.72±34.72	262.76±33.15

There was a significant increase in serum creatinine in the diabetic group and a significant decrease in the treated group when compared to the control group at $p<0.05$. There was a slight increase in diabetic and treated kidney creatinine compared to control rats. The liver creatinine of the diabetic was significantly higher $p<0.05$ compared is the control and treated group.

TABLE 5

Mean tissue total protein concentration at 540 nm in mg/ml(g/dl) for n=5

parameter	Control	Diabetic	Treated
Kidney protein mg/ml	5.47±3.18	5.94±2.48	5.89±2.17
Liver protein mg/ml	7.05±3.0	2.88±0.95	7.08±3.59

There was no significant difference P>0.05 in the kidney protein concentration in the groups . There was a significant decrease in liver protein concentrationP<0.05 of the diabetic rats compared to the control and the treated rats.

TABLE 6

Mean tissue sodium concentration at 550 nm in mEq/L for n = 5

Parameter	Control	Diabetic	Treated
Kidney sodium mEq/L	238.90±98.54	133.83±30.98	103.41±30.44
Liver mEq/L	156.55±29.97	86.18±32.80	82.41±20.88

There was no significant difference in the kidney sodium concentration p<0.05 of the diabetic when compared to control but the kidney sodium concentration of the treated group was significantly

decreased when compared to the control group at p<0.05. There was a significant decrease in liver sodium concentration of the diabetic and treated group when compared to the control at p<0.05.

TABLE 7

Mean tissue chloride concentration at 480nm in mg/L for n=5.

Parameter	Control	Diabetic	Treated
Serum chloride mEq/L	96.58±1.13	113.27±11.63	70.99±16.34
Kidney chloride mEq/L.	125.80±7.40	121.06±3.46	129.84±2.20
Liver chloride mEq/L	131.52 ± 9.87	135.79 ± 5.98	134.70 ± 3.16.

There was a significant increase in serum chloride in the diabetic compared to the control and the treated subjects at P <0.05. There was no significant difference in the kidney chloride of the diabetic and control group. There was significant increase in kidney chloride of the treated when compared with diabetic at p<0.05. There was no difference in the liver chloride concentration among the groups.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

Hyperglycemia is caused by impaired transport and uptake of glucose from the blood into both muscle and adipose tissue (Hallfisch and Shieldon 1981). Capillary lesions as a result of diabetic microangiopathy impair diffusion of factors that can stimulate synthesis and output of pancreatic juice ingredients (Goi and Lambardo 1987).

Type 2 diabetes mellitus is a public health problem of epidemic dimension and its prevalence is on the rise (Wild *et al* 2004). Many risk factors, including the metabolic syndrome have been implicated in its development (Abuissa and Bell 2005).

In this study, rats with normal fasting blood glucose as reported by Bradley (1959) were used, that is in the range of 96-112mg/100ml. Glanil was used to treat diabetes and was found to be a good hypoglycemic drug and can alleviate some of the symptoms of diabetes though it was found to activate some liver enzymes.

Rising liver enzymes activity may be as a result of hepatic inflammation and metabolic syndrome. The liver plays an important role in maintaining normal glucose concentration. It is also a major site of insulin clearance (Michael and Postic 2000).

Various tissues have been suggested as main source of plasma alkaline phosphatase including the liver (Ettinger and Feldman 2000) and alkaline phosphatase is an enzyme associated with plasma membranes (Wright *et al* 1972).

In this study, there was a significant increase in the serum, kidney and liver alkaline phosphatase activity of the diabetic compared to the control rats. This finding is in consonance with the previous report of increased total alkaline phosphatase activities in diabetes mellitus by (Street and Highman 1971, Maxwell and Hemie 1986, Bell and Thompson 1988, Thompson and Mikhailidia 1992, Cantor and Tuba 1997) .

In the experiment of Cantor *et al* 1997, there was a decrease in alkaline phosphatase activity in the 6 hours after alloxan administration, followed by a return to normal in 12 hours, then an elevation above normal by the second day and in forth day the activity was doubled its normal value, with further gradual increase in two to three weeks. Increased serum alkaline phosphatase activity were considered important evidence supporting the diagnosis of diabetes mellitus (Stewart 1991).

In acute pancreatitis, amylase levels are elevated for long periods (Wallenfels 1978). Amylase is produced in exocrine pancreatic cells, which is an adequate indicator of the organ's activity both in physiological and pathological states. In this study, serum amylase activity was increased in the diabetic rats and decreased in the treated rats showing that glanil reduced the progression of increased amylase activity in diabetes.

Serum creatinine is a useful tool in assessment of renal function. There was a significant increase in serum creatinine in the diabetic rats as compared to the control, and the treated groups showed reduced concentration of serum creatinine which indicates

that glanil can alleviate the problem of increased serum creatinine in diabetic subjects.

Hyperglycemia induced the overproduction of oxygen free radicals and consequently increased the protein oxidation (Coomes 1997). Increased protein oxidation causes reduced plasma total protein. There was no difference in the kidney total protein in this study which could indicate no proteolysis in the kidney. There was a significant decrease in the liver total protein concentration of the diabetic rats when compared to the control which shows that proteolysis could had occurred in diabetic liver. There was a significant increase in the liver total protein concentration $P<0.05$ of the treated group when compared to that in diabetic rats which indicates that glanil can alleviate the problem of proteolysis in the diabetic liver.

There was a significant increase in the serum chloride concentration of the diabetic rats compared to that of the control and treated groups which could show that glanil could treat increased serum chloride in the diabetics.

In conclusion from the results of this study, it may be speculated that monitoring the activities of alkaline phosphatase, and amylase or the concentration of creatinine, protein, chloride and sodium could help in diagnosing diabetes mellitus and also monitoring the condition or progress of the disease. It was also observed that glanil is a good hypoglycemic drug that could be used in the treatment and management of diabetes mellitus It is suggested

that more studies should be done on the hypoglycemic effect of glanil.

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APPENDIX 1

Table 1

Concentration of blood glucose level before alloxan administration (mg/100ml)

RATS	CONTROL	DIABETIC	TREATED
1	101	88	96
2	96	104	98
3	98	110	102
4	101	87	98
5	105	89	110

TABLE 2.

Final blood glucose concentration (mg/100ml)

RATS	CONTROL	DIABETIC	TREATED
1	101	305	97
2	96	269	73
3	98	280	83
4	101	310	100
5	105		

TABLE 3.

Serum alkaline phosphatase activity in μM/min/ml

RATS	CONTROL	DIABETIC	TREATED
1	2.33	7.01	6.15
2	2.99	6.68	11.07
3	3.32	6.68	7.28
4	3.32	5.65	-
5	3.65	-	-

TABLE 4

Kidney alkaline phosphatase activity in μM /min/mg protein

RATS	CONTROL	DIABETIC	TREATED
1	8.91	9.18	0.85
2	3.66	7.43	0.27
3	5.82	10.07	0.35
4	1.53	8.19	0.31
5	7.66	-	-

TABLE 5

Liver alkaline phosphatase activity in μM /min/mg protein

RATS	CONTROL	DIABETIC	TREATED
1	7.34	10.62	3.53
2	1.44	13.48	2.52
3	1.49	5.85	8.61
4	1.53	4.00	1.45
5	4.69	-	-

TABLE 6.

Serum alpha amylase activity in 1U/L.

RATS	CONTROL	DIABETIC	TREATED
1	41.97	135.07	123.2
2	62.71	148.24	115.78
3	53.06	112.26	149.54
4	48.24	167.54	174.90
5	96.48	-	-

TABLE 7

Kidney alpha-amylase activity in 1U/L.

RATS	CONTROL	DIABETIC	TREATED
1	101.30	98.94	115.26
2	77.18	67.54	115.78
3	19.30	74.12	107.43
4	41.97	38.59	32.34

5	4.82	-	-

TABLE 8.

Liver alpha amylase activity in 1U/L.

RATS	CONTROL	DIABETIC	TREATED
1	62.71	49.85	40.04
2	53.06	28.94	135.07
3	38.59	48.24	172.75
4	13.02	14.47	185.92
5	149.54	-	-

TABLE 9

Serum creatinine concentration in µmol/L.

RATS	CONTROL	DIABETIC	TREATED
1	506.72	679.30	270.98
2	446.42	507.50	206.76
3	412.74	591.30	223.20
4	405.05	699.80	111.98
5	590.50	-	-

TABLE 10

Kidney creatinine concentration in µmol /L.

RATS	CONTROL	DIABETIC	TREATED
1	53.26	101.2	24.28
2	50.12	96.25	133.14
3	61.87	68.50	112.78
4	97.12	98.80	107.26
5	83.80	-	-

TABLE 11

Liver creatinine concentration in µmol/L.

RATS	CONTROL	DIABETIC	TREATED
1	363.38	735.41	288.21
2	166.82	1096.00	137.84
3	192.66	708.00	302.30
4	204.41	211.46	322.67
5	473.04	-	-

TABLE 12

Kidney total protein concentration in g/dl (mg/ml).

RATS	CONTROL	DIABETIC	TREATED
1	5.29	9.06	2.94
2	1.41	2.24	8.12
3	10.59	6.99	7.80

4	3.06	5.47	4.65
5	6.99	-	-

TABLE 13

Liver total protein concentration in mg/ml.

RATS	CONTROL	DIABETIC	TREATED
1	3.47	2.82	3.53
2	12.41	4.29	10.29
3	5.12	2.82	3.47
4	6.94	1.59	11.06
5	7.29	-	-

TABLE 14

Kidney sodium concentration in mEg/L.

RATS	CONTROL	DIABETIC	TREATED
1	416.17	355.20	152.38
2	243.49	24.54	80.28
3	241.26	51.30	75.68
4	138.40	104.28	105.30
5	155.20	-	-

TABLE 15.

Liver sodium concentration in mEg/L.

RATS	CONTROL	DIABETIC	TREATED
1	127.88	97.58	80.38
2	206.88	100.37	101.58
3	144.61	30.67	48.68
4	146.84	116.09	97.80
5	-	-	-

TABLE 16.

Serum chloride concentration in mEg/L.

RATS	CONTROL	DIABETIC	TREATED
1	95.45	121.92	99.15
2	97.72	127.43	58.92
3	-	103.55	62.95
4	-	100.18	62.95
5	-	-	-

TABLE 17

Kidney chloride concentration in mEq/L.

RATS	CONTROL	DIABETIC	TREATED

1	116.70	123.53	129.32
2	126.80	125.38	130.59
3	130.36	117.50	132.78
4	118.55	117.84	126.67
5	136.62	-	-

TABLE 18.

Liver chloride concentration in mEg/L.

RATS	CONTROL	DIABETIC	TREATED
1	128.36	140.00	134.10
2	113.70	125.56	130.60
3	140.00	140.00	139.47
4	135.53	137.60	134.63
5	140.00	-	-

APPENDIX 2

Comparing the mean in the control and diabetic using student t-test.

Parameter	Degree of Freedom n-1	Table t-value	control against diabetic	P value	R.
Blood glucose	4	2.132	20.52	P<0.05	S
Serum ALP	4	2.132	10.90	P<0.05	S
Kidney ALP	4	2.132	2.50	P<0.05	S
Liver ALP	4	2.132	2.40	P<0.05	S
Serum amylase	4	2.132	8.43	P<0.05	S
Kidney amylase	4	2.132	1.08	P<0.05	NS
Liver amylase	4	2.132	1.29	P<0.05	NS
Serum Protein	4	2.132	-	-	-
Kidney protein	4	2.132	0.25	P<0.05	NS
Liver protein	4	2.132	2.94	P<0.05	S
Serum creatinine	4	2.132	2.99	P<0.05	S
Kidney	4	2.132	2.09	P<0.05	NS

creatinine					
Liver creatinine	4	2.132	2.46	P<0.05	S
Kidney chloride	4	2.132	1.27	P<0.05	NS
Liver chloride	4	2.132	0.80	P<0.05	NS
Serum chloride	4	2.132	2.86	P<0.05	S
Kidney sodium	4	2.132	1.35	P<0.05	NS
Liver sodium	4	2.132	3.34	P<0.05	S

APPENDIX 3

Comparing the mean of control and treated using student t- test.

Parameter	Degree of Freedom n-1	Table t-value	Control against treated	P value	R.
Blood glucose	4	2.132	2.01	P<0.05	NS
Serum ALP	4	2.132	4.73	P<0.05	S
Kidney ALP	4	2.132	4.27	P<0.05	S
Liver ALP	4	2.132	0.46	P<0.05	NS
Serum amylase	4	2.132	5.54	P<0.05	S
Kidney amylase	4	2.132	1.85	P<0.05	NS
Liver amylase	4	2.132	1.98	P<0.05	NS
Kidney protein	4	2.132	0.24	P<0.05	NS
Liver protein	4	2.132	0.01	P<0.05	NS
Serum	4	2.132	6.36	P<0.05	S

creatinine					
Kidney creatinine	4	2.132	1.13	P<0.05	NS
Liver creatinine	4	2.132	0.27	P<0.05	NS
Kidney chloride	4	2.132	1.16	P<0.05	NS
Liver chloride	4	2.132	0.68	P<0.05	NS
Serum chloride	4	2.132	3.13	P<0.05	S
Kidney sodium	4	2.132	2.95	P<0.05	S
Liver sodium	4	2.132	4.39	P<0.05	S

APPENDIX 4

Comparing the mean in the diabetic and treated using student t-test.

Parameter	Degree of Freedom n-1	Table t-value	Diabetic against treated	P value	R.
Blood glucose	3	2.353	20.11	P<0.05	S
Serum ALP	3	2.353	1.34	P<0.05	NS
Kidney ALP	3	2.353	16.56	P<0.05	S
Liver ALP	3	2.353	1.94	P<0.05	NS
Serum amylase	3	2.353	0.07	P<0.05	NS
Kidney amylase	3	2.353	1.12	P<0.05	NS
Liver amylase	3	2.353	3.33	P<0.05	S
Kidney protein	3	2.353	0.03	P<0.05	NS
Liver protein	3	2.353	2.26	P<0.05	NS
Serum creatinine	3	2.353	8.70	P<0.05	S

Kidney creatinine	3	2.353	0.14	P<0.05	NS
Liver creatinine	3	2.353	2.64	P<0.05	S
Kidney chloride	3	2.353	4.30	P<0.05	S
Liver chloride	3	2.353	0.32	P<0.05	NS
Serum chloride	3	2.353	4.23	P<0.05	S
Kidney sodium	3	2.353	0.45	P<0.05	NS
Liver sodium	3	2.353	0.21	P<0.05	NS

APPENDIX 5

Formulae.

$$\text{Mean} = \frac{\sum x}{N}$$

Where $\bar{x}$ = mean

$\sum$ = sum

x = observed values.

n = number of samples assayed.

$$\text{Standard deviation} = \sqrt{\frac{\sum (\bar{x} - x)^2}{N-1}}$$

Where SD = Standard deviation.

$\bar{x}$ = mean value for a particular set.

x = value for a single test.

$\sum (\bar{x}-x)^2$ = sum of the differences squared.

n = number of determination.

n-1 = one degree of freedom.

$$\text{Standard error of mean SEM} = \frac{SD}{\sqrt{n}}$$

Where SD = standard deviation.

$\sqrt{n}$ = square root of the number of sample assayed.

Student t-test.

$$t = \frac{\bar{x}_1 - x_2}{\sqrt{(SEM)_1^2 + (SEM)_2^2}}$$