EFFECTS OF OLEANOLIC ACID ON SMALL INTESTINE MORPHOLOGY AND ENZYMES OF GLUTAMINE METABOLISM IN DIABETIC RATS

BY

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BY

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Submitted in fulfillment of the requirements for the award of Masters of Science degree (research) in Biochemistry

School of Life Sciences

College of Agriculture, Engineering and Science

Supervisor: Dr B. Masola
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Student: Mr Murtala Isah Bindawa Signature ____________________

Supervisor: Dr B. Masola Signature ____________________
PRESENTATIONS

ABSTRACT

The small intestine (SI) is the main site for food absorption and glutamine utilization hence critical in metabolic disorders that involve energy balance such as diabetes and obesity. This study aims to assess the effects of a known hypoglycaemic compound, oleanolic acid (OA), on some enzymes of glutamine metabolism as well as morphological changes in the SI of diabetic rats. Diabetes was induced in male Sprague-Dawley rats by intraperitoneal injection of 60 and 40 mg/kg body weight streptozotocin for type 1 diabetes (T1D) and T2D respectively. One week later, different groups of the diabetic rats were treated with OA, insulin or OA + insulin in the T1D study and OA or metformin for T2D. Untreated diabetic groups served as controls and non-diabetic rats were grouped and treated similarly. After 2 weeks of treatment, all the animals were euthanized and the SI was differently processed for histological and enzymatic studies. The results in both studies indicate significant (p<0.05) increase in the size of the SI and villi in the diabetic compared to the non-diabetic groups and these were not ameliorated by all treatments. The results suggest that treatment with OA increases villi size independent of diabetes. On the other hand, the activity of phosphate dependent glutaminase (PDG) was slightly decreased only in T1D and this was not reversed by treatments. Expression of PDG detected by dot blots was not different in all groups. The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glutamate dehydrogenase (GDH) were significantly (p<0.05) elevated in diabetic control groups compared to normal controls and all treatments decreased ALT and AST activities while only insulin decreased GDH activity. Furthermore, hexokinase activity was elevated by diabetes and OA induces further increase. It is concluded that in the early stages of diabetes, OA treatment does not adversely affect PDG activity or expression in the SI but affects other enzymes important in energy metabolism in this tissue.
DEDICATION

This work is dedicated to my late father, Isah Ado Bindawa. May the blessing of Allah be with him.
ACKNOWLEDGEMENTS

I thank Almighty Allah for granting me life and health which are the foundation upon which the success of this work was built. I also thank Him for the gift of my mother and entire family who have tirelessly stood by me since childhood and still do.

I must acknowledge the invaluable support of my supervisor Dr. B Masola who has been my mentor, support and guidance since my coming to South Africa. It is an input I will use forever in my academic and scientific career.

This work wouldn’t have achieved its success without the efforts of technical staff from various units. The staff in the Biomedical Research Unit (BRU), Westville Campus, especially Dr Linda Bester, Mr. David Mompe and Rita Radebe deserve special mention. The help from Shoohana Singh of histology unit, Department of Physiology is beyond rewarding.

I will not forget the immense assistance I received from PhD and M Sc students of Dr Masola’s Laboratory. Namely: Andrew Mukundwa, Toluwani Tella, Ayodeji Oyenihi, Christie Manzini, Thobekile Dladla, Silvana Langa and Silungele Cele. Honours students Sindiswa Zondi and Mathabo Lutu have also been good friends in the lab.

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I must single out Dr. Auwal Ibrahim for his input in my academic life which is beyond describing. He has been relentlessly sowing the seeds of success in me and his contribution is a type I will reap in my entire living.

Finally my special appreciation to Prof. M.G. Abubakar who stood firmly by me during my study fellowship application to Umaru Musa Yaradua University. On that note, I am grateful to Umaru Musa Yaradua University and Education Trust Fund (ETF) Desk Office-UMYU for approving my fellowship at UKZN South Africa.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DC</td>
<td>Diabetic rats treated with solvent</td>
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<tr>
<td>DI</td>
<td>Diabetic rats treated with insulin</td>
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<tr>
<td>DM</td>
<td>Diabetic rats treated with metformin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DO</td>
<td>Diabetic rats treated with OA</td>
</tr>
<tr>
<td>DOI</td>
<td>Diabetic rats treated with OA + insulin</td>
</tr>
<tr>
<td>DPP-4</td>
<td>Dipeptidyl peptidase-4</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
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<td>FPG</td>
<td>Fasting plasma glucose</td>
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<td>GC</td>
<td>Goblet cells</td>
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<td>GDH</td>
<td>Glutamate dehydrogenase</td>
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<td>GDM</td>
<td>Gestational diabetes mellitus</td>
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<tr>
<td>GI</td>
<td>Gastrointestine</td>
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<td>GIS</td>
<td>Gastrointestinal system</td>
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<td>GIT</td>
<td>Gastrointestinal tract</td>
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<tr>
<td>GLP-1</td>
<td>Glucagon like peptide 1</td>
</tr>
<tr>
<td>GLP-2</td>
<td>Glucagon like peptide 2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>HK</td>
<td>Hexokinase</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>IDDM</td>
<td>Insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>IGT</td>
<td>Impaired glucose tolerance</td>
</tr>
<tr>
<td>kDA</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced form)</td>
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<tr>
<td>NC</td>
<td>Non-diabetic rats treated with solvent</td>
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<tr>
<td>NI</td>
<td>Non-diabetic rats treated with insulin</td>
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<tr>
<td>NIDDM</td>
<td>Non-insulin dependent diabetes mellitus</td>
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<td>NO</td>
<td>Non-diabetic rats treated with OA</td>
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<td>NOI</td>
<td>Non-diabetic rats treated with OA + insulin</td>
</tr>
<tr>
<td>OA</td>
<td>Oleanolic acid</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
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<tr>
<td>PDG</td>
<td>Phosphate dependent glutaminase</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RPG</td>
<td>Random plasma glucose</td>
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<tr>
<td>SI</td>
<td>Small intestine</td>
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<tr>
<td>STZ</td>
<td>Streptozotocin</td>
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<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
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<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
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CHAPTER ONE

Introduction and Literature Review

1.0 Diabetes mellitus (DM)

Diabetes mellitus is a term used to describe a group of metabolic disorders resulting from defects in either secretion or organ response to insulin and sometimes both, with a resulting characteristic hyperglycaemia. When left unmanaged, the chronic high blood glucose level leads to long-term pathologies of various organs especially the eyes, kidneys, central nervous system, heart and vascular system (ADA, 2013). The prevalence of the disease cuts across all age groups, sex and populations, and the number of reported cases is increasing due to the global rise in obesity, population growth, ageing and age-specific prevalence (Danaei et al., 2011). People with diabetes are at increased risk of developing cardiovascular, peripheral vascular and cerebrovascular diseases (Stratton et al., 2000) and have higher susceptibility to communicable diseases such as tuberculosis (Jeon and Murray, 2008). The strategic management of the condition is aimed at controlling the elevated glucose level and the resulting complications, through behavioural and therapeutic interventions (Stumvoll et al., 2005).

1.1 Epidemiology and economic burden of diabetes

The current prevalence of diabetes is already 8.3% corresponding to 382 million people living with diabetes globally and this number is estimated to rise to 592 million by the year 2035 (Guariguata et al., 2014b). This is a result of both change in the trend of diagnosis and increase in the incidence of the disease (Danaei et al., 2011). Regionally, Western Pacific has the largest number of people living with diabetes, corresponding to 132 million people while Middle
East/North Africa has the highest prevalence with 10.9%. Africa has the lowest of both where 15 million people live with diabetes equivalent to 4.5% prevalence (Whiting et al., 2011).

However, Africa where countries are low to middle income bears the highest mortality burden. Africa alongside other low and middle income countries in other regions account for as high as 80% of the deaths caused by diabetes!. Worldwide, diabetes resulted in 4.8 million deaths in 2012 (Whiting et al., 2011) and it is projected to be the 7th leading cause of all global deaths by 2030 (Mathers and Loncar, 2006). Diabetes is also associated with a huge economic burden. In 2011, annual cost of diabetic care globally was 471 billion US dollars (Whiting et al., 2011) which was 19% higher than was projected for 2025 (IDF, 2003). About 90% of these costs are spent in the economically richest countries while the low and middle-income countries accounted for only < 10%. Moreover, these costs also represent about 13% of the National health care budget expenditure on diabetes in 80% of the countries (Zhang et al., 2010). South Africa with a prevalence of 7.04% corresponding to close to 2 million adults living with diabetes spent approximately 1.4 billion dollars on diabetes in 2011 (Whiting et al., 2011).

1.2 Classification of diabetes

The basic classification of diabetes generally takes into account the differences in the etiology and/or onset of the disease. In this context diabetes mellitus is commonly classified into three types: Type I and II diabetes mellitus and gestational diabetes. Each of these types of diabetes can be diagnosed by measuring the fasting plasma glucose (FPG), random plasma glucose (RPG), oral glucose tolerance test (OGTT) or glycated haemoglobin (A1C) depending on the severity of the condition. An FPG of ≥ 126 mg/dL (7 mmol/L) or an RPG ≥ 200 mg/dl (11.1 mmol/L) is enough to diagnose an individual with classic symptoms of hyperglycaemia.
However, for an asymptomatic individual, at least one other FPG or RPG or alternatively an oral glucose tolerance test (OGTT) is needed to confirm diabetes (Albert and Zimmet, 1998). On the other hand an A1C value ≥ 6.5% reflects long term (usually 2-3 month) diabetes (ADA, 2013).

![Figure 1.1: Global Estimate of the number of people with diabetes in 2013 (20-79 years)](Adopted from IDF 2013)

1.2.1 **Type 1 diabetes (T1D)**

Type 1 diabetes or Insulin Dependent Diabetes Mellitus (IDDM) or juvenile onset diabetes affects 5-10% of people living with diabetes and mostly manifests in childhood (Guariguata *et al.*, 2014b). This type of diabetes is a result of an autoimmune destruction of pancreatic β-cells resulting in defects of insulin secretion (Tuomilehto, 2013). Hence type 1 diabetics inevitably rely on exogenous insulin for survival (Atkinson and Eisenbarth, 2001). Normally, the immune system differentiates between self and non-self cells in its defense action. However, in type 1 diabetes, genetic defects coupled to environmental factors lead to the self-destruction of β-cells.
by immune response which is eventually diagnosed as T1D (Daneman, 2006). This process can
be detected by markers, among which are islet cell autoantibodies and autoantibodies to the
insulin hormone (ADA, 2013). In humans the genes strongly associated with T1D include those
for human leukocytes antigen (HLA) and insulin among others (Acharjee et al., 2013). On the
other hand, the contribution of environmental factors to the development of T1D is still poorly
understood. However, geographical location, exposure to certain substances found in diets and
high glycaemic index diets seem to play crucial roles in the development of T1D (Acharjee et al.,
2013). Moreover, a nonstructural protein of coxsackievirus b4 is shown to trigger cytokines
release from T helper cells which are associated with development of T1D (Varela-Calvino et
al., 2000), suggesting a connection between parasitic, viral and bacterial infections with
increased risk of the disease (Herold et al., 2013).

1.2.2 Type 2 diabetes (T2D)

Also known as Non-Insulin Dependent Diabetes Mellitus (NIDDM) is the most common type of
diabetes comprising of approximately 90 % of all people with diabetes (Guariguata et al.,
2014b). Type 2 diabetes is characterized by both diminished organ sensitivity to insulin (insulin
resistance) and the islet’s β-cells inability to produce more compensatory insulin (Henriksen et
al., 2011).

The etiology of insulin resistance is complex, involving genetic predisposition, obesity,
sedentary life style, pregnancy and hormone excess (Evans et al., 2002). For instance, genetic
predisposition is reported to increase the risk for T2D by 2.4 fold and the chances of developing
T2D increases if one or both parents had the disease. Moreover, 15-20 % of the closest relatives
of a diabetic individual eventually develop a form of impaired glucose tolerance (IGT) (Stumvoll
et al., 2005). Also, the incidence of T2D strongly correlates with that of obesity (Figure 1.2) and sometimes the term “diabesity” is used to describe the relationship between the two conditions (Castagneto and Mingrone, 2012). Excess visceral and subcutaneous fat which characterizes obesity leads to increased circulating unesterified fatty acids that causes poor response to insulin especially in skeletal muscle and excess hepatic glucose production (Gastaldelli, 2011). This initial defect in insulin response by the skeletal muscle is critical in the development of early prediabetic impaired glucose tolerance which culminates in T2D (Leahy, 2005). Non-obese Type 2 diabetic patients on the other hand may begin with a deficiency in early insulin secretion after meal leading to abnormal level of free fatty acids and glucagon responses which further develops to diabetes (Bruce et al., 1988).

Elevated blood glucose level associated with T2D (as well as other types) leads to oxidative stress (OS), protein glycation and autoxidation of glucose. Furthermore, oxidative stress is particularly important in the etiology of insulin resistance (Evans et al., 2002). Causatively, OS has been shown to be partly a result of increased mitochondrial hydrogen peroxide production induced by elevated free fatty acids and NADPH oxidase activation (Henriksen et al., 2011). This early consequences of hyperglycaemia result in progressive defects of β-cell function leading to the characteristic poor insulin secretion observed in T2D (Porte and Kahn, 2001).

1.2.3 Gestational diabetes mellitus (GDM)

Gestational diabetes mellitus refers to any degree of glucose intolerance first recognized during pregnancy. The condition is continually becoming an important global issue with about 50% of women with early GDM going on to develop type 2 diabetes within 5-10 years of giving birth. Moreover, about 21.4 million GDM cases out of an estimate 127.1 million live births in 2013
were reported (Guariguata et al., 2014a). Gestational diabetes results in pregnancy complications (ADA, 2013) and infants of GDM patients tend to be more prone to overweight or obesity (Guariguata et al., 2014a).

**Figure 1.2:** Relationship between obesity and insulin resistance (adopted from Gastaldelli, 2011).

Key: FFA = free fatty acids, VLDL = very low density lipoprotein, GLP-1 = glucagon like peptide 1, GNG = gluconeogenesis, ↑ = increase

### 1.3 Glucose homeostasis

Homeostasis is the general term used to refer to the body’s internal mechanism for maintaining viable environment for proper well-being. This involves maintaining optimum range of factors such as the internal temperature, pH, proteins, oxygen, mineral elements and blood glucose
concentration (Schulkin, 2003). Maintaining an optimum level of plasma glucose is a complex mechanism that involves crosslink among different metabolic pathways of glucose production vis-à-vis consumption in various organs. This homeostatic strategy is of particular importance for the brain, red blood cells and some tissues of the eye that rely solely on glucose as an energy source for normal metabolic functions (El Bacha et al., 2010). Consequently, the plasma concentration of glucose in humans is remarkably maintained at 4.5-5.2 mmol/L and rarely falls below 4 mmol/L or rises above 7 mmol/L. The fall of glucose below 4 mmol/L or hypoglycaemia, leads to confusion, convulsion, loss of consciousness and eventually death. Similarly, acute/chronic elevation of plasma glucose which is the main characteristic of diabetes mellitus is equally devastating (Marshall and Bangert, 2008).

Plasma glucose is derived from the diet and absorbed into the blood stream via the gut or produced in tissues. Diet derived glucose may amount to 3000 mmol (540 g) in a 24 hr period. This large amount of glucose is taken up for immediate metabolism by tissues such as the central nervous system (CNS) (which requires about 30 mmol/hr supply of glucose) and skeletal muscle (Berg et al., 2002). Some tissues such as the liver and skeletal muscle further deposit excess glucose as glycogen in the process of glycogenesis and once the glycogen pool is filled, glucose may then be converted into fats in the liver and adipose tissues (Marshall and Bangert, 2008).

In the post-absorptive state, stored glycogen is mobilized via glycogenolysis for internal energy demands and that in the liver for replenishing the plasma pool. The ability of the liver to contribute to plasma glucose is a result of the presence of the enzyme, glucose-6-phosphatase, that catalyzes the final step in the production of free glucose from glucose-6-phosphate (Barzilai and Rossetti, 1993). After the exhaustion of the glycogen pool, for example under prolonged starvation or diabetes, other metabolites such as lactate and amino acids provide carbon skeleton
for glucose production via gluconeogenesis. Once again, in these states the liver alongside the kidney (under very long starvation) and more recently the small intestine are the known gluconeogenic organs that contribute to plasma glucose concentration (Barzilai and Rossetti, 1993; Mithieux et al., 2004). Apart from their function in glucose production, the kidneys may serve to dispose of high glucose levels in to the urine, a condition known as glucosuria. This is one of the earliest important symptoms of individuals with chronic diabetes mellitus (Marshall and Bangert, 2008).

Glucose balance is under tight regulation by hormonal and chemical signals (Lam et al., 2009), like most homeostatic processes in the body. Among the hormones that are known to have effect on glucose homeostasis in normal and diseased individuals are the corticosteroids, leptins and most importantly the insulin/glucagon ratio (McKay and Cidlowski, 2003; Morton and Schwartz, 2011; Steiner et al., 1973). Glucocorticoids (corticosteroids) produced by the adrenal cortex affect transcriptional and enzymatic processes involved in gluconeogenesis and glycogenesis (McKay and Cidlowski, 2003) while adipose tissue-derived leptin acts via the central nervous system to maintain energy balance (Morton and Schwartz, 2011). On the other hand, insulin in concert with glucagon also affect multiple signaling pathways important in glucose metabolism and balance (Marshall and Bangert, 2008). Impaired action of one or more of these hormones may result in hypoglycaemia, glucose intolerance and diseases associated with poor energy balance such as obesity and diabetes mellitus.

1.3.1 Insulin signaling

Insulin is an α,β-dimer of 21 and 30 amino acids polypeptides linked by two disulfide linkages and synthesized in the β-cells of the pancreatic islets (Brange and Langkjær, 1993). The
secretion of the hormone is triggered by increase in blood glucose level, for example after a carbohydrate rich meal. Once released, insulin causes receptor mediated transmembrane transport of glucose into cells (Figure 1.3) of insulin sensitive tissues which include white adipose tissue, brown adipose tissue and skeletal muscle (Way et al., 2001). This occurs in concert with changes in glucagon secretion and entails the body’s regulatory mechanism for blood glucose balance (Steiner et al., 1973).

![Figure 1.3: Insulin signalling cascade (adopted from Abdul-Ghani and DeFronzo 2010)](image)

Key: IRS-1 = insulin receptor substrate 1, PI-3-kinase = phosphatidylinositol 3-kinase, p85 = 85 kDa subunit of PI-3-K, p110 = 110 kDa subunit of PI-3-K, Akt = protein kinase B/Akt, NOS = nitric oxide synthase, GLUT 4 = glucose transporter 4.

Insulin binding to extracellular α-subunit of IRS-1 causes phosphorylation of multiple tyrosine residues on transmembrane β-subunit of IRS-1. The phosphorylated tyrosine residues mediate an activating association between 85 kDa regulatory subunit and 110 kDa catalytic subunit of PI-3-K. Activation of PI-3-K in turn leads to activation of Akt which initiates the processes involved in the translocation of GLUT 4 containing vesicles to the plasma membrane. Finally GLUT 4 acts as the major transporter of glucose from
the extracellular to the intracellular compartment of insulin sensitive tissues. Insulin also regulates protein, lipid and glycogen synthesis via the activated PI-3-K as well as activation of nitric oxide synthase. Nitric oxide synthase generates nitric oxide in the endothelium with protective consequences on the endothelial lining (Abdul-Ghani and DeFronzo, 2010).

Insulin possesses positive regulatory effects on the transcription of glycolytic enzymes which include glucokinase and phosphofructokinase and negative effect on phosphoenolpyruvate carboxykinase which is gluconeogenic. Hence, when any of the many factors that impair insulin release or organ response occurs, disturbance in body’s glucose homeostasis results which manifests in early impaired glucose tolerance (IGT) and consequently diabetes mellitus (Leahy, 2005).

Glucagon unlike insulin is a single polypeptide of 29 amino acids secreted from α-cells of the pancreas. The insulin:glucagon ratio determines the anabolic-catabolic setting in an organism. As a result, both insulin and glucagon have other regulatory effects on nutrients metabolism apart from the maintenance of optimum blood glucose level (Jaspan and Rubenstein, 1977).

1.4 Pathophysiology of diabetes

Diabetes is accompanied by increased risk of many microvascular and macrovascular diseases. Although the pathogenesis of type 1 and type 2 diabetes are different, the pathophysiology of their resulting vascular complications is similar (van Dijk and Berl, 2004). The main cause of these complications is the hyperglycaemia associated with both type 1 and type 2 diabetes which selectively damages cells found in the endothelium and mesangial cells (Brownlee, 2005). Thus, most of the observed effects of high blood glucose levels affect virtually all the small blood
vessels found in organs throughout the body (Dokken, 2008) especially the eyes, kidneys and nerves (Brownlee, 2005). This is basically due to the inability of the cells found in these organs to reduce the in-flux of elevated blood glucose concentration. Approximately one third to half of the people with diabetes are affected with diabetes-induced organ damage as a result of the microvascular (retinopathy, neuropathy and nephropathy), and macrovascular (cardiovascular disease, cerebrovascular diseases and peripheral vascular diseases) complications. Factors that increase the risk of developing diabetes complications include; hyperglycaemia, duration of diabetes, age, tobacco use, hypertension and dyslipidemia (Cade, 2008).

An underlying way through which high blood glucose leads to these pathological changes has been identified to be hyperglycaemia-induced increase in reactive oxygen species (ROS). Increased ROS such as superoxide leads to inhibition of glyceraldehydes-3-phosphahate dehydrogenase (GAPDH) by activating poly (ADP-ribose) polymerase (PARP) (Brownlee, 2005). Accumulation of upstream glycolytic metabolites as the result of GAPDH inhibition activates the pathways that are central to the pathology of diabetes. These activations include: increase flux in the polyol pathway, increased formation of advanced glycation end products (AGEs), induced activation of protein kinase C (PKC) and increased flux into hexosamine pathway (Brownlee, 2001). Superoxide can also interact with nitric oxide produced by nitric oxide synthase isozymes found in the endothelium. This leads to generation of peroxynitrite, which is a strong DNA and protein damaging oxidant, leading to increased cardiovascular dysfunction (Pacher and Szabó, 2006). However, hyperglycaemia does not directly lead to macrovascular damage observed in diabetes. Rather, the damage was proposed to initiate from a different mechanism which in the end leads to activation of the four pathways as for microvascular damage. This mechanism is the increased flux of free fatty acids (FFA) from
adipocytes to the macrovascular endothelium. Subsequent β-oxidation of the FFA and oxidation of the acetyl-coA produced through the process increase intracellular electron donors that in turn increase ROS and the eventual macrovascular damage (Brownlee, 2005).

1.5 Management and treatment of diabetes

Management of T1D and T2D differ in relation to the different etiologies of the conditions. Because T1D involves destruction of β-cells, the patients inevitably rely on exogenous insulin for survival. However, combined therapy with other agents that reduce the resulting pathologies, such as cardiovascular complications, is often implemented. Insulin treatment of type 1 diabetes involves subcutaneous doses of insulin at intervals before and after meal depending on the type of the regimen and the age of the individual (Shulman and Daneman, 2010). However, the treatment requires the patient and/or his family to master the procedures involved such as the insulin injection, glucose monitoring and recommended nutritional plan. Different approaches are employed for the delivery of the insulin regimens which may involve combination of basal bolus with either daily doses of insulin injections or insulin pumps. This is often tricky and sometimes the treatment involves the risk of developing hypoglycaemia even when appropriately followed. Fortunately, advances in diabetes care allows for easier hormone delivery and simpler glucose monitoring (Atkinson and Eisenbarth, 2001; Van Belle et al., 2011). Moreover, various forms of recombinant human insulin with better pharmacokinetics than the conventional insulin are now commercially available in some parts of the world (Atkinson and Eisenbarth, 2001).

On the other hand, different classes of drugs are in use for the management and treatment of T2D. The main aim of the therapeutic intervention is to ameliorate glucose tolerance or at least prevent the long term effects of hyperglycaemia. As a result, many drugs are targeted to improve
the organ sensitivity to insulin because of its central role in the pathogenesis of the disease (Stumvoll et al., 2005). Although exogenous insulin cannot be a rationale treatment for type 2 diabetes, increasing the level of circulating insulin is sometimes one of the strategies in improving the efficacy of insulin sensitizers. Hence, low doses of insulin are often used in a combination therapy with drugs used to treat T2D such as metformin (Stumvoll et al., 2005).

Several other classes of drugs have been developed over the years each with a unique mechanism of action. However, almost all of these drugs are not without one form of side effect or another (Table 1.1). Among these drugs are the thiazolidinediones which improve tissue sensitivity to insulin (Stumvoll et al., 2005). The main site of action of these drugs is the adipocytes, where the receptor, peroxisome proliferator-activator receptor gamma (PPARγ) is highly expressed. Activation of PPARγ modulates free fatty acid metabolism and redistribution as well as adipokines secretion. Furthermore, the associated reduction in the circulating tumor necrosis factor α and increase in adiponectin cytokines with these drugs improves insulin sensitivity in the peripheral tissues (Hauner, 2002).

Another class of popular antidiabetic drugs are the biguanides of which metformin is currently in use (Cheng and Fantus, 2005). Metformin is an effective anti-hyperglycaemic drug that acts independent of the pancreas (Stumvoll et al., 2005). Despite the long use of metformin clinically, its mechanisms of action are still not fully elucidated. However, decrease intestinal glucose absorption, plasma free fatty acids and liver gluconeogenesis, and increase glucose delivery to cells are described to be some of the ways through which metformin imposes its observed anti-hyperglycaemic effects (Grzybowska et al., 2010).
Acarbose, an α-glucosidase inhibitor, has also been an effective anti-hyperglycaemic drug. It inhibits di saccharidases found in the proximal intestine which are responsible for the breakdown of dietary oligosaccharides and disaccharides. Therefore, acarbose essentially delays the intestinal absorption of carbohydrate and consequently reduces postprandial blood glucose levels. The efficacy of this class of drugs is less than that of most oral anti-hyperglycaemic agents (OHAs), hence they are mostly used clinically in combination with other OHAs (Lebovitz, 1997).

Some anti-diabetic drugs exert their action by binding directly to receptors found on the pancreas. Sulfonylureas basically improve insulin secretion from the β-cells through binding to their receptor that is intimately associated with an ATP sensitive potassium channel found on the membrane of the cells (Panten et al., 1996). Binding of sulfonylureas to the receptor-channel complex causes membrane depolarization of resting β-cells via inhibition of cross-membrane efflux of potassium. This depolarization further results in the opening of calcium channels, facilitating influx of calcium which causes the exocytosis of insulin from storage vesicles (Cheng and Fantus, 2005). Given that this action is irrespective of the blood glucose levels, it is possible to develop hypoglycaemia with the treatment.

The treatment approach that appears to take into account the blood glucose level involves the incretin hormones which are secreted from distal small intestine. Glucagon-like peptide 1(GLP-1) possesses a glucose-dependent insulinotrophic effect as well as β-cell trophic and intestinal motility inhibitory effects which collectively result in reduced blood glucose levels (Stumvoll et al., 2005). However, the circulating hormone is vigorously inactivated by dipeptidyl peptidase-4 (DPP-4), making it therapeutically impossible to use exogenous GLP-1 like insulin. Thus, GLP-1
analogues resistant to DPP-4 and DPP-4 inhibitors are used for the treatment of T2D clinically (Namba et al., 2013).

Recently, surgical procedures have emerged to be the safe and effective treatments for T2D (Sala et al., 2012). Originally, bariatric surgery such as gastric banding was used for the treatment of obesity. However, it was later experienced that obese patients with T2D recover from diabetes after the surgery in parallel with weight loss. This recovery from diabetes was initially solely attributed to the weight loss after gastric banding, which is essentially a surgical insertion of a plastic band in the stomach to limit food intake (Castagneto and Mingrone, 2012). Subsequently, the observation that a robust glycaemic control occurs with more advanced surgical procedures such as the gastric bypass suggested other mechanisms independent of weight loss (Cohen et al., 2011). Gastric bypass involves excluding some length of the small intestine from food transit, resulting in lower nutrient absorption among other effects. This raised interest on the importance of the small intestine in glucose homeostasis especially during diabetes (Mithieux, 2009). The proposed mechanisms for the anti-diabetic effects of bariatric surgeries include; stimulation of intestinal gluconeogenesis, increase secretion of insulinotrophic hormones and inhibition of anti-incretins from the foregut (Sala et al., 2012).

Other surgeries such as pancreas and islets transplantation have also emerged as surgical cures for type 1 diabetes. However, transplantation must be accompanied by immunosuppressive drug treatment to prevent rejection and/or recurrent autoimmune destruction of the transplants. Moreover, lack of organ donors is another major obstacle to the procedure (Atkinson and Eisenbarth, 2001). When successful, pancreas transplant results in exogenous insulin independence and improvement of cardiac and cardiovascular risk factors (Coppelli et al., 2003).
as well as reduction in proteinuria associated with early diabetic nephropathy (Paleologo et al., 2004).

1.5.1 Management of other diabetic complications

T2D is associated with increased risk of hypertension, cerebrovascular diseases and coronary artery diseases. Hence, management of other complications alongside glycaemic control is beneficial. Angiotensin converting enzyme (ACE) inhibitors and statins are respectively associated with decrease risk of hypertension and dyslipidemia in diabetics. Drugs associated with decrease risk of atherosclerosis such as aspirin are also recommended as a secondary therapy during diabetes (Stumvoll et al., 2005). Physical therapy may also be used to reduce the risk of microvascular and macrovascular diseases through the physical management of obesity, pain and poor endurance. In achieving that, monitoring the exercise of diabetic patients is especially important (Cade, 2008).

1.6 The gastrointestinal system (GIS)

The gastrointestinal system essentially consists of the gastrointestinal tract and the associated glands. The gastrointestinal tract is the tube that runs from the oral cavity, through the esophagus, stomach, small and large intestine, rectum, and the anus and is further categorized into the upper and lower gastrointestinal tract. The upper gastrointestinal tract starts from the oral cavity down to the small intestine and performs functions such as the transport of digested food, enzymatic secretion, nutrients digestion and absorption, and protection against the external environment (Treuting et al., 2012). Digestion of ingested food is initiated by enzymes secreted in the mouth and continued by gastric secretions with minimal absorptions taking place (DeSesso and Jacobson, 2001). On the other hand, the small intestine is the major site of nutrient digestion.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Example of drug</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exogenous insulin</td>
<td>• Rapid action</td>
<td>• Risk of hypoglycaemia</td>
<td>Rapid acting, short acting, pre-mixed</td>
<td>Shulman and Daneman, 2010</td>
</tr>
<tr>
<td></td>
<td>• Treatment for type 1 diabetes</td>
<td>• Very technical</td>
<td>insulin</td>
<td></td>
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<td></td>
<td></td>
<td>• Invasiveness</td>
<td></td>
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<tr>
<td>Thiazolidinediones</td>
<td>• Improvement of vascular abnormalities</td>
<td>• Weight gain</td>
<td>Pioglitazone, rosiglitazone</td>
<td>Cheng and Fentus, 2005</td>
</tr>
<tr>
<td></td>
<td>• Amelioration of dyslipidemia</td>
<td>• Anemia</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>• Edema</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>• Cognitive heart failure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biguanides</td>
<td>• No weight gain</td>
<td>• Abdominal discomfort</td>
<td>Metformin, phenformin (withdrawn from</td>
<td>Stumvoll et al., 2005;</td>
</tr>
<tr>
<td></td>
<td>• Improvement of lipid profile</td>
<td>• Diarrhea</td>
<td>market due to toxic effects)</td>
<td>Cheng and Fentus, 2005</td>
</tr>
<tr>
<td></td>
<td>• Decrease cardiovascular risks</td>
<td>• Anorexia</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>• Bloating</td>
<td></td>
<td></td>
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<tr>
<td>α-glucosidase inhibitors</td>
<td>• Good safety profile,</td>
<td>• Flatulence</td>
<td>Acarbose, miglitol, voglibose</td>
<td>Lebovitz, 1997; Cheng and</td>
</tr>
<tr>
<td></td>
<td>• No risk of hypoglycaemia</td>
<td>• Abdominal discomfort</td>
<td></td>
<td>Fentus, 2005</td>
</tr>
<tr>
<td></td>
<td>• Decrease post-prandial plasma triacylglyceride levels</td>
<td>• Bloating</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Successful glycaemic control</td>
<td>• Diarrhea</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Decrease microvascular and macrovascular complications</td>
<td>• Bloating</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfonylureas</td>
<td>• Successful glycaemic control</td>
<td>• Risk of hypoglycaemia</td>
<td>Glebenclamine, glimepiride, tolbutamide,</td>
<td>Stumvoll et al., 2005</td>
</tr>
<tr>
<td></td>
<td>• Decrease microvascular and macrovascular complications</td>
<td>• Risk of renal failure</td>
<td>tolibutamide, glipizide, tolamamide</td>
<td></td>
</tr>
<tr>
<td>Incretin based therapy</td>
<td>• Glucose dependent action</td>
<td>• Inactivation by DPP-IV</td>
<td>Exenatide, lixisenatide, liraglutide,</td>
<td>Chrysant and Chrysant, 2012</td>
</tr>
<tr>
<td></td>
<td>• Cardiovascular protective effect</td>
<td></td>
<td>dulaglutide, albiglutide,</td>
<td></td>
</tr>
<tr>
<td>Surgical procedures</td>
<td>• No risks of drug complications</td>
<td>• Maximal invasiveness</td>
<td>Gastric bypass, duodenoejejunectomy,</td>
<td>(Ahn et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>• Robust results</td>
<td>• Immune compromise (in case of islets</td>
<td>pancreas and Islet transplants</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>transplants</td>
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</table>
and absorption due to its anatomical and physiological adaptations (Reed and Wickham, 2009). Although the GIT represents only about 5% of the total body mass, it accounts for approximately 20% of total oxygen consumption of the body due to the high energy demand as the lining GIT is renewed and the high activity of transmembrane transporters (Blachier et al., 2009). Consequently, the GIS is paramount in the energy homeostasis in mammals and a strong relationship exists between the gastrointestinal motor and sensory functions and overall blood glucose control (Rayner et al., 2001). Hence, this link is particularly important in disorders that involves energy metabolism such as diabetes mellitus.

1.6.1 The small intestine (SI)

The SI deserves particular consideration because of the critical function it performs in blood glucose homeostasis. These functions include digestion of food, nutrients absorption, and secretion of hormones. In humans, small intestine accounts for about 81% of the total length of the approximately 835 cm intestinal canal. The SI consists of three subdivisions which are the duodenum (25 cm), jejunum (260 cm) and ileum (395 cm). Similarly, in rats the SI runs an average length of 125 cm which is 83% of the total intestinal canal comprising of 9.5-10 cm duodenum, 90-135 cm jejunum and 2.5-3.5 cm ileum (DeSesso and Jacobson, 2001).

After the passage of chyme (partially digested food) from the stomach to the duodenum, enzymes originating from the stomach, intestine and pancreas as well as bile acids are secreted to continue with the digestion in the duodenum (Reed and Wickham, 2009). Moreover, absorption of nutrients largely occurs in the duodenum and the proximal jejunum which is enhanced by some permanent spiral or circular folds termed the plicae circulares found on the intestinal lining or mucosa (Figure 1.4). The surface area of the mucosa is further enhanced by the villi (singular: villus) and crypts which are fingerlike projections and depressions respectively as well as smaller microvilli. Furthermore, the epithelium of the villi and crypts is lined by a continuous sheet of absorptive enterocytes. Additionally, each villus
has an artery, a vein and a specialized lymphatic channel that absorbs and transport nutrients called the lacteal. Absorbed carbohydrates (and proteins) or lipids enter the circulation via the portal blood or are secreted into lymph as chylomicron particles respectively (Kohan et al., 2011). Outside the epithelial mucosa lies a layer of connective tissue consisting of blood and lymphatic vessels called the lamina propria. The lamina propria absorb and deliver nutrients as well as transport hormones secreted by endocrine cells found on the endothelium (Reed and Wickham, 2009). Moreover, goblet cells are found throughout the length of the small intestine. These cells synthesize and secret mucins which forms the protective mucous membrane of the small intestine (Specian and Oliver, 1991).

**Figure 1.4:** Internal structure of the small intestine (adopted from OpenStax, 2013)

### 1.7 Role of the small intestine in glucose homeostasis

Although glucose is not the main metabolic fuel for the small intestine, the organ is central to glucose homeostasis. Recently, organs other than the liver, muscle and pancreas have been receiving more attention in the attempt to elucidate the etiology, pathogenesis and treatments of diabetes mellitus (Maggs et al., 2008). The gastrointestinal tract in general is continuously viewed as an important component of glucose homeostasis especially in a diabetic state. The
“portal glucose signal” is a known phenomenon that influences several aspects of glucose homeostasis via a neural-mediated mechanism involving hepatoporal vagal and spinal afferents (Mithieux, 2010). This alongside other signals prevents endogenous glucose production (EGP) in a fed state via the suppression of glucagon and stimulation of insulin release. Moreover, the gastrointestinal tract becomes the major source of blood glucose after feeding (Aronoff et al., 2004) and this is key to determining postprandial glucose flux into the circulation. Interestingly, the rate limiting step of “gastric emptying” is not affected by either insulin or glucagon (Maggs et al., 2008). Rather, the delivery of eaten food from the stomach to the duodenum is affected by several other hormones involved in glucose regulation, some of which are of intestinal origin. These hormones include amylin from the pancreas, and glucose-dependent insulinotrophic peptide (GIP) and glucagon like peptide 1 (GLP-1) secreted by the L-cells of the small intestine (Aronoff et al., 2004). The intestine further regulates energy metabolism by sensing the accumulation of long chain fatty acid which is a signal to the gut-brain axis through the nervous system to decrease satiety and hepatic glucose production (Breen et al., 2011).

During a fasting state, the plasma glucose concentration is a function of the rate of endogenous glucose production (EGP) by the liver and its uptake by other tissues of the body (Maggs et al., 2008). To achieve adequate EGP in this state, the hormone glucagon, released from the pancreatic α-cells stimulates hepatic glucose production (HGP), via glycogenolysis or gluconeogenesis during a short or long fasting respectively (Aronoff et al., 2004). Moreover, there are increasing evidences that, the small intestine expresses glucose-6-phosphatase, the enzyme necessary for the release of glucose into the circulation (Croset et al., 2001), and contributes to blood glucose substantially during long fasting and diabetes (Mithieux et al., 2004). Also, the SI has been shown to deposit glycogen synthesized from absorbed glucose and this property has been utilized as a target for glucose disposal for
conditions such as diabetes (Khathi et al., 2013). As mentioned previously, surgeries that target the small intestine have been successful in ameliorating glucose tolerance in diabetes, further suggesting a special role of the small intestine in glucose homeostasis (Rubino, 2008).

When glucose is utilized in the small intestine, the first step of its conversion is catalyzed by the enzyme; hexokinase (EC 2.7.1.1). Hexokinase catalyzes the phosphorylation of glucose (and other hexoses) using ATP as phosphoryl donor generating glucose-6-phosphate (Figure 1.5) as product (Wilson, 2003). Four distinct isozymes that play different roles have been identified in mammals namely; type I, type II, type III and type IV (also referred to as glucose kinase due to its high $K_m$ for glucose). Furthermore, the small intestine has been shown to express type I and type II HK as major types as well as type III and IV in lesser quantity (Weiser et al., 1971). Hexokinase has been shown to be the rate limiting enzymes for glycolysis in the small intestinal mucosa and hence critical in glucose utilization. The activity of HK has been shown to be altered under different feeding conditions, aging and metabolic disorders (Jones and Mayer, 1973; Srivastava and Hubscher, 1968). Glucose feeding increased HK activity while starvation was observed to decrease the activity in both mitochondrial and soluble preparations of rat jejunum. Diabetes on the other hand increased the activity of soluble form of hexokinase while decreasing the mitochondrial bound HK signifying release of mitochondrial bound HK (Anderson and King, 1975).

1.8 Glutamine metabolism in the SI

Glutamine is the most abundant plasma amino acid that performs many functions in different organs which include acting as: an oxidative fuel for intestine; the major gluconeogenic precursor for the liver; substrate for the synthesis of protein, urea, nucleotides and nucleic acids; inter-organ nitrogen transport; precursor for neurotransmitter; and source of ammonia in renal acid-base homeostasis. (Newsholme et al., 2003).
Figure 1.5: Phosphorylation of glucose catalyzed by hexokinase as the first step of common pathways of glucose metabolism

Glutamine alongside the ketone bodies has been identified as the major fuel sources for the small intestine (Table 1.2). Apart from its function as an energy source, glutamine is important physiologically in the prevention of intestinal injury (Chang et al., 2001) and general maintenance of gut integrity (Newsholme et al., 2003). The SI is responsible for the removal of almost the entire glutamine produced from other organs, mainly skeletal muscle, suggesting a large influx of glutamine (Schrock and Goldstein, 1981). However glutamine accounts for approximately 35 % only of the total carbon (IV) oxide (CO$_2$) production by the SI under normal metabolic state (Windmueller and Spaeth, 1978). The remainder of the glutamine serves as a precursor for lactate and other amino acids such as alanine, citrulline, proline and ornithine (Mithieux, 2001).

A different role of glutamine is activated during fasting and diabetes. In these states, the intestinal net glucose release via gluconeogenesis becomes important and glutamine serves as the major precursor. In addition, the associated portal release of alanine contributes to hepatic
gluconeogenesis, thus intestinal glutamine utilization during fasting and diabetes is critical in the overall glucose homeostasis (Mithieux, 2001).

Table 1.2: Contribution of arterial metabolites to intestinal CO\(_2\) production (Windmueller and Spaeth, 1978)

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine</td>
<td>35</td>
</tr>
<tr>
<td>2-hydroxybutyrate</td>
<td>26.1</td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>24.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>6.8</td>
</tr>
<tr>
<td>Lactate</td>
<td>4.6</td>
</tr>
<tr>
<td>Unesterified fatty acids</td>
<td>3.4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

1.8.1 Phosphate dependent glutaminase (PDG) in the small intestine

Glutaminase (EC 3.5.1.2) catalyzes the oxidative deamidation of glutamine to yield glutamate and ammonia. Two forms of glutaminases with strong similarity in amino acid sequence identity are known: the hepatic type which is found only in the liver and the kidney type which has wider organ distribution (McCauley et al., 1999). Apart from the difference in organ distribution, another major variation between the hepatic and renal isoforms is their pattern of regulation. While the hepatic type is affected by nutritional status such as diabetes, high protein diet and fasting, the kidney type appears to be regulated by acid-base status only (Watford, 1993). The kidney type glutaminase is also highly active as well as the major glutamine metabolizing enzyme in the SI. The enzyme is phosphate dependent and associated with the mitochondria of the enterocytes found along both the villus and crypt of duodenum, jejunum and ileum. Moreover, the intestinal phosphate dependent glutaminase (PDG)
resembles the kidney type 72 kDA glutaminase in terms of expression (Pinkus and Windmueller, 1977). However, the intestinal glutaminase unlike the kidney type is not activated by acidosis. The SI glutaminase is rather activated by phosphate ($P_i$), bicarbonate and sulphate but inhibited by ammonium ions (Masola and Zvinavashe, 2003). Maximum activity of the enzyme was obtained at $P_i$ concentration of 10 mM at pH 9 (Masola and Ngubane, 2010). In the same study the stability of mitochondrial membrane has been shown to be pivotal for the activity of the enzyme. Furthermore, ADP, AMP and ATP nucleotides activate the enzymes with ADP being the highest activator (Masola and Ngubane, 2010). A concentration of 100 mM of phosphate is necessary for the dimerization and activation of isolated glutaminase. Moreover, glutamate is a feedback inhibitor of the enzyme among other inhibitors (McCauley et al., 1999). Other factors that increase the activity of the enzyme include weaning (Hahn et al., 1998), lactation (Ardawi, 1987) and presence of glucocorticoids (Sarantos et al., 1992), and increased liver cirrhosis (Romero-Gómez et al., 2004) while sepsis (Ardawi et al., 1990), interleukins and interferon-$\gamma$ (Austgen et al.1992; Sarantos et al., 1994) cause decrease in activity. Furthermore, starvation (Kong et al., 2000) and glucocorticoids (Sarantos et al., 1992) increase the expression of small intestinal glutaminase. Intestinal PDG is important because it catalyzes the first reaction that commits carbon skeleton of glutamine to energy production, protein synthesis or a more recently discovered function of the SI, gluconeogenesis, depending on the metabolic condition (Croset et al., 2001). During prolonged diabetes, the activity of PDG in the small intestine is increased up to 40 % which makes it crucial in the overall glucose homeostasis during diabetes (Mithieux et al., 2004).

1.8.2 Glutamate dehydrogenase (GDH) in the small intestine

Glutamate dehydrogenase (GDH) (EC 1.4.1.3) is a mitochondrial matrix enzyme that catalyzes the reversible oxidative deamination of glutamate to $\alpha$-ketoglutarate ($\alpha$-KG)
utilizing NAD\(^+\) or NADP\(^+\) as a coenzyme (Fang et al., 2002; Smith and Stanley, 2008). In mammals, the enzyme consists of six identical subunits of approximately 56 kDa and 550 amino acids each and is found in various tissues including the liver, brain, pancreatic islet, kidney and intestine (Mastorodemos et al., 2005). The regulation of GDH is tight with the major activators being ADP and leucine while GTP and ATP being the major inhibitors (Li et al., 2012). The direction of either glutamate production or deamination by GDH depends on the redox potential of the mitochondria. At high NADH/NAD\(^+\) ratio such as at normal fed state, reductive transamination of α-KG is favored thereby yielding glutamate as the cataplerotic product. Conversely, at low NADH/NAD\(^+\) ratio flux in the α-KG direction is favored (Frigerio et al., 2008).

A mutation in GDH gene whereby the GTP binding site is altered leads to hyper-activation of the enzyme leading to leucine dependent excessive increase in β-cell insulin release. The resulting hyperinsulinism is associated with excess amount of ammonia a condition known as hyperinsulinism/hyperammonemia syndrome observed in infants and children with a characteristic hypoglycaemia (Fang et al., 2002).

**1.8.3 Alanine aminotransferase and aspartate aminotransferase in the SI**

Alanine aminotransferase (ALT) (EC 2.6.1.2) and aspartate aminotransferase (AST) (EC 2.6.1.1) play a major role in the interplay between glucose and amino acids metabolism in the body (Sookoian and Pirola, 2012). The enzymes have wide tissue distribution and perform vital functions in liver, kidney and small intestine where the activities of the enzymes are high (Kobayashi et al., 2010). In the intestine, the metabolic fate of glutamine and glutamate is not entirely oxidation. Although most of the dietary and endogenous glutamine as well as glutamate undergo oxidation to CO\(_2\) in the intestine, the remaining are converted to lactate and other amino acids and released in to the portal circulation (Burrin and Stoll, 2009).
main route for the conversion of glutamate to α-KG in the enterocytes is through transamination reaction because of the low capacity of these cells for glutamate dehydrogenase reaction (Blachier et al., 2009). This transamination, catalyzed by either ALT or AST occurs in the presence of pyruvate or oxaloacetate, to yield α-KG and alanine or aspartate respectively.

![Metabolic pathways cross links between glutamine and glucose during diabetes](image)

**Figure 1.6:** Metabolic pathways cross links between glutamine and glucose during diabetes (adapted from Mithieux, 2001)

Key: plain lines = routes of glutamine carbons, broken lines = routes of glucose carbons (major routes in bold), Ala = alanine, α-KG = α-ketoglutarate, Glc6P = glucose-6-phosphate, Glc6Pase = glucose-6-phosphatase, Gln = glutamine, Glu = glutamate, G3P = glycerol-3-phosphate, Lac = lactate, LDH = lactate dehydrogenase, ME = malic enzyme, OAA = oxaloacetate, OAADC = oxaloacetate decarboxylase, PC = pyruvate carboxylase, PEP = phosphoenolpyruvate, PEPCK = phosphoenolpyruvate carboxykinase, P\(_i\) = inorganic phosphate, Pyr = pyruvate, TCA = tricarboxylic acid.
The serum activities of the enzymes are routinely used clinically as markers of liver damage. However, abnormal levels of the serum level of the enzymes is also associated with increased risk of metabolic syndrome including type 2 diabetes (Sookoian and Pirola, 2012). Moreover, decreased serum activity of ALT was observed to correlate with small intestinal ALT activities in rats maintained under intermittent feeding. Hence, in addition to liver injury, the activities of the enzymes in tissues is altered by hormonal and nutritional modifications (Kobayashi et al., 2010). ALT in addition plays a central role in linking glutamine and glucose metabolism in the small intestine (Figure 1.6), hence critical in energy utilization of the organ (Mithieux, 2001).

1.9 Diabetes induced and morphological and metabolic changes in the small intestine

1.9.1 Morphological changes in the small intestine during diabetes

Increase in weight of the small intestine resulting from diabetes was recorded as early as 8 days after induction in rats. This rose up progressively up to 140 days after induction at which the wet of the SI may be doubled that of normal rats and this is about four times when expressed per body weight. Increase intestinal length (16.6 %) and circumference (3.6 %) were also recorded by around 6 weeks after diabetes induction in rats. The increased weight was found to be predominantly in the mucosal scraping rather than the underlying tissue (Schedl and Wilson, 1971). Moreover, microscopic examination of diabetic SI also showed various forms of pathological abnormalities. In a study using diabetic Chinese hamsters, the villi were observed to be deformed and longer (10.7 %) with concomitant excessive lesions (13-53 %), lymphocytes aggregations (6-40 %) and elevated number of goblet cells (11-33 %) (Diani et al., 1976). Moreover, diabetes has been found to increase only the number of goblet cells but the intracellular mucin content of the SI was decreased. Furthermore, a decrease in crypts depth has been noted to occur late in diabetes (Mantle et al., 1989). On the
other hand, the underlying musculis interna, muscularis externa (14-47 %) and Auerbach plexuses (14-47 %) were reduced in diabetic animals compared to normal hamsters (Diani et al., 1976). However, contrasting data on the muscular thickness was obtained with experimental type 1 diabetic rats. Zhao et al. (2003) reported a marked increase (up to 20 %) in all of mucosal, submucosal and total wall thickness from day 7 up to 4 weeks after induction of STZ-type 1 model of diabetes. Further findings from the study are that opening angle and residual strain which reflect the elastic properties of the SI were lower in the duodenum and higher in the jejunum and ileum of diabetic rats compared to non-diabetic rats. Subsequent findings by the same research group showed the observation to be true of type 2-diabetic model Goto-Kakizaki (GK) rats and is positively correlated with the level of blood glucose and advanced glycation end products (AGEs) (Zhao et al., 2013). Contrary to these findings, similar study on the tension-strain relations found no difference in mucosa, submucosa and muscle layer thickness in experimental diabetic rats injected with lower STZ (28 mg/kg body weight) dose after 28 days. In agreement however, the study also suggested increase in wall stiffness in the jejunum and ileum of the diabetic rats (Jørgensen et al., 2001). The disparity in the result may be due to the differences in the resulting rise in blood glucose levels. The latter study reported just around half the blood glucose level of the previous study further suggesting a relationship between the morphomechanical changes to blood glucose levels. The authors suggest these observed changes that affect both longitudinal and circumferential stiffness of the SI to be in part responsible for the abdominal discomforts commonly complaint by diabetic patients (Zhao et al., 2003).

Morphological changes in the small intestine appear to be a result of multiple physiological and metabolic changes. Increased intestinal growth in diabetes has been shown to correlate positively with increased hyperphagia which is undoubtedly a compensatory mechanism for the glucose loss in the urine (Thulesen et al., 1999). Additional mechanism however will be
the reported increase in GLP-2 which possesses an intestine trophic effect. Immuno
normalization of the GLP-2 resulted in restoration of increased muscularis surface area
observed in the proximal intestine of diabetic control animals (Hartmann et al., 2002). GLP-2
has been shown to have no effect on food intake in experimental animals (Tsai et al., 1997).
However plasma GLP-2 was shown to correlate with other factors that influence upper
intestinal growth in diabetes such as dietary fiber (Thulesen et al., 1999). These findings
suggest an influence of hyperphagia on GLP-2 secretion, both of which synergistically
increase intestinal growth. Inhibition of apoptosis has also been suggested to be one
explanation for the observed increase in villi length and mucosal thickness in experimental
diabetes (Noda et al., 2001). Whether the same factors are responsible for both lengthening of
the villi and the whole intestine remain to be investigated.

1.9.2 Metabolic changes in the small intestine during diabetes

1.9.2.1 Glucose absorption and metabolism

Diabetes mellitus is accompanied by changes in absorption and motor function of the small
intestine (Ebert, 2005; Fujita et al., 1998). The accompanying increase in postprandial
glycaemia is continuously recognized as the major contributor to overall glycaemic control in
type 2 diabetes (Ma et al., 2009). This increase in absorption is a result of the changes that
occur at the brush border membrane (BBM) and basolateral membranes (BM) of the SI due
to rise in the activity, mRNA and protein level of sodium glucose transport protein (SGLT1),
Na⁺/K⁺-ATPase and glucose transporter 2 (GLUT2) (Fujita et al., 1998, Li et al., 2013).
Furthermore, diabetic patients that develop neuropathic diabetes tend to have accelerated
gastric emptying which correlate with accelerated intestinal absorption (Frank et al., 1995).
Consequently, delaying gastric emptying for instance by opiate analgesics or prokinetic
agents such as morphine resulted in decrease postprandial plasma glucose concentration in
type 2 diabetics (Gonlachanvit et al., 2003). As discussed previously, a special function of the small intestine, intestinal gluconeogenesis, is activated during diabetes and may contribute up to 35% of total indigenous glucose production (Mithieux et al., 2004). Hence changes in intestinal glucose metabolism are important aspect of diabetic complications.

1.9.2.2 Cholesterol and other lipids

During diabetes, cholesterol biosynthesis is increased in all the segments of the small intestine. The increase in intestinal cholesterol biosynthesis in the early phase of diabetes may be attributed to increase in the activity of enzymes for the cholesterol synthesis. However, at later stages, the increase is mainly due to the hypertrophy of the small intestine (Feingold and Moser, 1984). Furthermore, cholesterol synthesis is increased more in the distal segment than the proximal segment of the small intestine. All the cells types on the villus-crypt axis in the distal segment show this increase but is more pronounced in the cells found on the upper villus (Feingold and Moser, 1987). Moreover, conditions that lead to increase food intake such as obesity and pregnancy also lead to increased intestinal cholesterol synthesis (Feingold et al., 1986). This suggests that increased food intake associated with uncontrolled diabetes is the main stimulus for increased cholesterol synthesis. Not only was the level of indigenous synthesis observed to increase during diabetes, but also the rate of absorption and transport of dietary cholesterol. Further, the synthesis of intestinal triacylglycerols and transport via hepatic very low density lipoproteins (VLDL) into the circulation are also increased during diabetes. This increase in the transport of lipids from the small intestine in the form of chylomicrons may initiate changes in the lipid metabolism which may be important in atherosclerotic vascular disorders (Feingold, 1989). Collectively these changes in lipid metabolism constitute the central pathophysiological feature of insulin resistance and T2D (Levy et al., 2010).
1.9.2.3 Hormonal secretion

The role of the small intestine in blood glucose regulation was recognized after the observation that oral glucose caused a four times increase in blood glucose than intravenous glucose (DeFronzo et al., 1978). This led to the discovery of the gastrointestinal hormones responsible for the glucose dependent insulin secretion known as the incretin effect. The two known incretin hormones are glucose dependent insulinotropic hormone (GIP) secreted by the K-cells of the proximal small intestine and glucagon like peptide secreted by the L-cell of the distal ileum (Kazakos, 2011). The incretin effect has been shown to decrease during diabetes due to decrease action of GIP and reduced secretion of GLP-1 (Nauck et al., 1993a). The decreased action of GIP is attributed to the reversible β-cells insensitivity to circulating GIP which may be caused by hyperglycaemia. Because the action of GLP-1 is preserved in type 2 diabetes, continuous subcutaneous injection of the hormone was deemed to be an important therapeutic strategy (Nauck et al., 1993b). However as stated earlier, the action of both GIP and GLP-1 is short lived due to their inactivation by dipeptidylpeptidase (DPP-4) making the only alternative therapeutic strategy in incretin based therapies being either GLP-1 agonists or DPP-4 inhibitors (Kazakos, 2011).

1.10 OLEANOLIC ACID (OA)

Oleanolic acid (3b-hydroxyolean-12-en-28-oic acid) (Figure 1.7) belongs to pentacyclic triterpenes class of biologically active compounds. It is highly ubiquitous and has been isolated from more than 1500 plant species, among which many are used as routine source of food and medicine. However, high content of OA in olive plant (Olea europae; family: oleaceae) serves as the major commercial source. OA exists in plants both as the free acid and part of glycoconjugates (saponins) that serve a defense function to the plants (Pollier and Goossens, 2012).
The compound is increasingly attracting attention due to its various pharmacologic actions. These include hepatoprotective effects, antioxidant, anti-inflammatory, anti-cancer and anti-diabetic activities. The compound is clinically used alone or in combination with other drugs in the treatment of acute liver injury, chronic fibrosis and cirrhosis of the liver (Liu, 2005). Furthermore, OA has been shown by many researchers to be crucial in the management of diabetes mellitus (Castellano et al., 2013). The compound was shown to prevent visceral adiposity with a resulting improvement in glucose tolerance (de Melo et al., 2010). Oleanolic acid also improves organ sensitivity to insulin (Wang et al., 2013) and possesses antiglycative (Wang et al., 2010) and cardioprotective (Mapanga et al., 2012) properties in diabetic rats. Furthermore, OA inhibits the inflammatory and specific immune responses associated with islet allograft, thereby prolonging the survival of the cells (Nataraju et al., 2009). Thus, the compound is of great interest to researchers as a possible candidate for the treatment of diseases especially diabetes.

1.10.1 Mechanism of antihyperglycaemic action of oleanolic acid

The hypoglycaemic properties of OA are also a result of its ability to prevent excess glucose absorption and production by the gut; increase organ sensitivity to insulin; and protect β-cells.
Oleanolic acid was shown to inhibit liver gluconeogenesis and increase hepatic insulin sensitivity (Wang et al., 2011). The insulin sensitizer effects of OA can be partly explained by its ability to modulate insulin signaling cascade via protein tyrosine phosphatase 1B (PTP-1B), which is an enzyme that deactivates insulin receptor. Oleanolic acid binds to near the catalytic site of PTP-1B and in doing so inhibiting the enzyme and essentially increasing tissue insulin sensitivity (Ramírez-Espinosa et al., 2011). The antidiabetic actions of OA are also related to the ability of the compound to interfere with important processes in the etiology of diabetes such as oxidative stress, dyslipidemia and inflammatory responses. Wang et al. (2013) demonstrated that, OA stabilizes nuclear factor erythroid 2 p45-related factor 2 (Nrf2) which is a crucial regulator in maintaining the pool of antioxidant enzymes. Moreover, the serum triglycerides, total cholesterol (TC), LDL-cholesterol and free fatty acids (FFAs) as well as the level of proinflammatory factors were shown to be reduced with OA treatment in type 2 diabetic rats (Wang et al., 2013). Treatment with OA also inhibits the small intestine uptake of glucose with a concomitant increase in glycogen biosynthesis in the intestinal wall. This may play a crucial role in decreasing postprandial glycaemia (Khathi et al., 2013). The β-cell-protective effects of OA against lipotoxicity and apoptosis is also important in maintaining normal insulin response to blood glucose level (Wang et al., 2011). Consequently, OA is also associated with decrease in diabetes associated pathologies such as myocardial infarction (Mapanga et al., 2012) and renal failure (Wang et al., 2010).
1.11 RESEARCH JUSTIFICATION

Diabetes is unarguably a morbid disease that needs to be controlled. Unfortunately, the pathogenesis and physiological changes that are associated with the disease are still not fully understood. Oleanolic acid has been shown to possess hypoglycaemic effects through different mechanisms. Further investigations are still needed to explore the full potential of the phytochemical. The specialized aspects of glutamine/glutamate metabolism of different glutamine-utilizing cells and the relationship of aspects of metabolism to cell function have been reported elsewhere (Newsholme et al., 2003). Hence elucidation of the correlation between oleanolic acid and glutamine metabolism in small intestine, the organ that utilizes the metabolite most vigorously, will give additional information on overall energy utilization in diabetic condition. This is fundamental to management of the disease.

1.12 RESEARCH OBJECTIVES

1.12.1 General Objective

This research is aimed at investigating the effects of oleanolic acid on small intestine morphology and glutamine metabolizing enzymes in the small intestine of type 1 and type 2 diabetic rats.

1.12.2 Specific Objectives

a) To investigate the effects of oleanolic acid on histopathological changes occurring in the small intestine during diabetes.

b) To investigate the effects of oral administration of oleanolic acid on the activities of phosphate dependent glutaminase, glutamate dehydrogenase, alanine aminotransferase and aspartate aminotransferase in the small intestine of streptozotocin induced type 1 and type 2 diabetic rats.

c) To determine the changes in the expression of glutaminase after OA treatment.

d) To deduce possible relationship between histopathological changes and glutamine metabolism.
CHAPTER TWO

Materials and Methods

2.1 Chemicals and reagents

Oleanolic acid (OA) was obtained from Shaanxi King Stone Enterprise, China. Streptozotocin, metformin, enzymes (L-glutamate dehydrogenase, lactate dehydrogenase and malate dehydrogenase all in buffered 50 % glycerol), amino acids, α-ketoglutarate, citric acid, sodium citrate, potassium phosphate dibasic (K$_2$HPO$_4$), potassium phosphate monobasic (KH$_2$PO$_4$), hydrochloric acid, TRIZMA base, potassium chloride, PMSF, benzamidine, bovine serum albumin (BSA), hydrazine, β-nicotinamide adenine dinucleotide (NAD$^+$), β-nicotinamide adenine dinucleotide reduced form (NADH), adenosine-5'-triphosphate (ATP), adenosine-5'-diphosphate (ADP), perchloric acid, triethanolamine hydrochloride (TEA), ammonium acetate, EDTA, DMSO, formalin, xylene, hematoxylin dye and BCPI/NBT premixed liquid substrate were purchased from Sigma, St Loius, MO, USA. Eosin dye, copper (II) sulphate pentahydrate (CuSO$_4$·5H$_2$O) and potassium sodium tartarate (KNaC$_4$H$_7$O$_4$·H$_2$O) were obtained from SAARCHEM, Krugersdorp, South Africa. Absolute ethanol, potassium hydroxide and potassium iodide were obtained from Merck Millipore, Darmstadt, Germany. All SDS-PAGE chemicals and secondary antibody (goat anti-rabbit IgG alkaline phosphatase conjugate were obtained from Bio-Rad, Hercules, California, USA. Primary antibodies anti-rabbit IgG kidney-type glutaminase antibody (KGA/PDG) was from Santa Cruz Biotech USA and anti-rabbit IgG β-actin antibody which acted as the loading control was purchased from BioLegend, San Diego, USA. Insulin (Humalog KwiPen) was purchased from Eli Lilly and Company, USA.
2.2 Animals

Male Sprague-Dawley rats (200-250 g body weight) were procured from the Biomedical Resource Unit (BRU) at Westville Campus of the University of KwaZulu-Natal, South Africa. Drinking water and feed (commercially available rat chow diet) were provided for the animals ad libitum for the period of the experiment. Handling and maintenance of the animals were according to the rules and regulations of the animal ethics committee of the University (Ethics approval reference: 001/14/Animal. See appendix 1).

2.3 Animals Grouping, Induction of Diabetes and Treatment

The animals for the studies were randomly grouped as depicted in Figures 2.1A and 2.1B for type 1 and type 2 diabetes studies respectively.

Diabetes was induced by a single intraperitoneal injection of streptozotocin dissolved in freshly prepared 0.1 M citrate buffer pH 4.5 at doses of 60 mg/kg body weight or 40 mg/kg bw for type 1 or type 2 respectively. Control animals were injected with the buffer only. One week after the treatment, blood glucose concentrations were determined. A fasting blood glucose concentration of 18 mmol/L or above was considered as a stable diabetic state for type 1 diabetes whereas concentrations of 7-14 mM/L was considered as a stable diabetic state for type 2 diabetes. The blood for glucose determination was collected from the tail vein of the rats (approximately 50 µl) and measured using Glucometer (Accu-check performa nano, USA).

All treatment drugs at the stated doses (Figure 2.1A and 2.1B) were dissolved in 50 % DMSO and administered orally except for insulin which was subcutaneous. The treatments lasted for fourteen days after which the animals were sacrificed using halothane anesthesia and the whole small intestine was excised, weighed, measured (length) and prepared as described below.
2.4 Experiment design

**Figure 2.1**: Flow chart for studies on (A) type 1 diabetes (B) type 2 diabetes.

**KEY**: STZ = streptozotocin  
N = non-diabetic  
D = diabetic  
C = solvent treated  
O = oleanolic acid treated  
I = insulin treated  
M = metformin treated
2.5 Intestine sampling

The abdomen of the sacrificed rats was immediately cut open, and the small intestine removed from the abdomen cavity. Duodenum was considered as first 10 cm beyond the pylorus. The middle 15 cm of the remaining segment was obtained as the jejunum and the bottom 3.5 cm as the ileum (DeSesso and Jacobson, 2001). The intestinal sections were then rinsed using physiological saline solution and 1 cm of each part was preserved in 10% formalin. The rest of the jejunum was frozen at liquid nitrogen temperature and stored at -20°C until use.

Prior to enzyme assays, the frozen intestines were thawed and homogenized (5 ml/mg tissue) in buffer (50 mM Tris and 120 mM KCl, 0.5 mM PMSF and 5 mM benzamidine pH 7.4) using Omni homogenizer, USA.

2.6 Enzyme assays

2.6.1 Phosphate dependent glutaminase (PDG)

Preparations: Buffer A (10 mM K₂HPO₄, 50 mM TRIZMA base, 120 mM KCl, pH 8.6), buffer B (50 mM Tris, 310 mM hydrazine, pH 9).

The enzyme was assayed at 37°C as described by Curthoys and Weiss (1974) with modifications by Masola and Zvinavashe, (2003). The two step procedure involves the determination of the amount of glutamate formed by PDG using glutamate dehydrogenase (GDH) (Figure 2.2). The reaction mixture contains the following in final concentrations; freshly prepared 20 mM glutamine (in buffer A) and 2 mM NAD⁺ + 0.25 mM ADP (in buffer B). 50 µl sample was added to 200 µl buffer A with glutamine except for the blank which contains equal volume of buffer only. The mixture was incubated for 15 min at 37°C. The reaction was terminated by deproteinization with 120 µl of 30% perchloric acid and the tubes
vortexed and placed on ice for 5 min. 225 µl KOH was subsequently added and the mixture brought to room temperature before adding 2 ml buffer B (with freshly added NAD⁺ + ADP). The mixture was centrifuged at 3000 rpm for 5 min. Oxidation of glutamate was initiated by the addition of 10 µl GDH (13 units) in 50 % glycerol and incubating for 45 min at room temperature. The amount of NAD⁺ reduced to NADH was estimated spectrophotometrically at 340 nm using Varian Cary 50 UV/Vis Spectrophotometer, SMM Instruments Midrand, South Africa. Calculations of enzyme activity were carried out using a NADH millimolar extinction coefficient of 6.22 M⁻¹cm⁻¹.

**Figure 2.2:** Assay of Phosphate dependent glutaminase (PDG) activity

### 2.6.2 Glutamate dehydrogenase (GDH), aspartate aminotransferase (ALT) and alanine aminotransferase (AST)

The activities of GDH, AST and ALT were determined at 25 °C as adapted by Masola *et al.* (2008).

GDH activity was estimated in the direction of glutamate formation (Figure 2.3) by following the oxidation of NADH spectrophotometrically at 340 nm (Schmidt, 1974). The reaction mixture contains the following in final concentrations: buffer mixture (50 mM triethanolamine hydrochloride/100 mM...
Figure 2.3: Assay of glutamate dehydrogenase (GDH) activity

ammonium acetate/2.5 mM EDTA, pH 8), freshly prepared mixture of 1 mM ADP/0.2 mM NADH/8.6 units ml⁻¹ lactate dehydrogenase and 0.2 ml of sample. The absorbance is then read at 340 nm to the total consumption of the pyruvate. Thereafter, the reaction is started by the addition of 7 mM α-ketoglutarate in buffer in a final volume of 3 ml.

Figure 2.4: Assay of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity

ALT (AST) activity was determined by coupling the reaction to the reduction of generated pyruvate (oxaloacetate) by lactate dehydrogenase (malic dehydrogenase) as shown in Figure 2.4. The rate of oxidation of NADH in the reduction reaction is measured at 340 nm (Volmann-Mitchell and Parsons, 1974). The reaction mixtures contain the following in final concentrations: 0.1 M sodium phosphate buffer pH 7.6; 7 mM α-ketoglutarate in buffer; 0.2 mM NADH in buffer; 8.6 units/ml L-lactic dehydrogenase (LDH) (or 19 units/ml malic
dehydrogenase [MDH] for AST) in 50 % glycerol. This is followed by the addition of 0.1 ml sample. Non-specific oxidation of NADH is then recorded. The reaction is thereafter started by the addition of 33 mM L-alanine (or L-aspartate for AST) in buffer in a final reaction volume of 3 ml.

Specific activity of GDH, ALT and AST are expressed in nmoles of NADH oxidized per min per mg sample protein taking the millimolar extinction coefficient of NADH at 340 nm as 6.22 M⁻¹cm⁻¹.

2.6.3 Hexokinase (HK)

Hexokinase activity was determined at 30 °C using the method described by Goward et al. (1986) with modifications by Ngubane et al. (2011). The assay involves estimation of glucose-6-phosphahate produced by HK as it is oxidized by glucose-6-phosphate dehydrogenase with concomitant reduction of nicotinamide adenine dinucleotide phosphate (NADP⁺) (Figure 2.5). The reaction mixture contains the following in final concentration: 20 mM TEA-HCl buffer (pH 7.6); glucose (222 mM); MgCl₂ (8 mM); NADP⁺ (0.91 mM); ATP (0.64 mM); mercaptoethanol (5 mM) and 0.55 Uml⁻¹ glucose-6-phosphate dehydrogenase. The reaction was initiated by the addition of 100 µl sample and the appearance of NADPH was followed spectrophotometrically for 10 min at 340 nm.

![Diagram of hexokinase activity](image)

Figure 2.5: Assay of hexokinase activity.
2.7 SDS-PAGE and western blotting

Preparations: Sample buffer (v/v 32.5 % deionized water, 12.5 % 0.5 M Tris-HCl, 25 % glycerol, 20 % SDS, 2 % bromophenol + 0.5 % mercaptoethanol prior to use), running buffer (30.3 g Tris base, 144 g glycine, 10 g SDS diluted 1:10 prior to run), resolving gel (v/v 41 % deionized water, 33 % acrylamide/bisacrylamide, 25 % 1.5 M Tris-HCl buffer, 1 % SDS + 50 µl APS + 5 µ TEMED/10 ml), Stacking gel (v/v 41 % deionized water, 33 % acrylamide/bisacrylamide, 25 % 0.5 M Tris-HCl buffer, 1 % SDS + 50 µl APS + 10 µ TEMED/10 ml), butanol:water (1:1 v/v), fixative (40 % methanol + 7 % acetic acid), transfer buffer (25 mM Tris, 150 mM glycine, 20 % v/v methanol), TBS-tween buffer (200 mM Tris, 500 mM NaCl, 0.05 % Tween 20), blocking buffer (5 % w/v nonfat dried milk in TBS-tween buffer).

SDS-PAGE was performed using standard lab procedure for electrophoretic separation of proteins. Resolving gel was degassed and loaded into the wells, covered with butanol:water and allowed to polymerize for 45-60 min. After pouring away the butanol:water layer, the gel was washed with deionized water and dried. Degassed stacking gel was loaded and the comb positioned. The stacking gel was allowed to polymerize for 15-30 min after which the comb was removed and the gel washed and dried. The gel was placed in the electrophoretic chamber containing the running buffer. Protein samples (50 µg) and 5 µl of molecular marker were subsequently loaded and separated at 100-150 V (Mini-PROTEAN 3 Cell, Bio Rad. South Africa). After separation, the gels were used for staining or blotting.

The gel was fixed for 30 min and stained in commassie stain for 30-45 min on a rocker. The gel was then destained in the fixative overnight on the rocker.
2.7.1 Glutaminase blotting

The gel was sandwiched into the cassette in the following order starting from the black colour side: filter pad, filter paper, gel, nitrocellulose membrane, filter paper, filter pad. The proteins were then transferred from the gel to nitrocellulose membrane at 100 V for 2 hr in the electrophoresis chamber (Mini Trans-Blot Cell, Bio-Rad, South Africa) containing the transfer buffer. After the transfer was completed, the nitrocellulose membrane was placed in blocking buffer overnight at 4°C.

Primary antibody was diluted 1:500 (or 1:1000 depending on the primary antibody) in blocking buffer and overlaid on the nitrocellulose membrane and gently shaken for 1 hr at room temperature. The membrane was washed 3 times with TBS-tween buffer for 5 min each. The procedure was repeated with the secondary antibody except no blocking buffer was used in the final washing. Finally BCIP/NBT solution was added as the substrate and exposed until colour development and washed with deionized water.

For the dot blot analysis, 20 µg of proteins from each sample were spotted and allowed to air-dry. The blocking and addition of antibodies were 30 min each while the washing step was same as above. The bands were visualized using a Syngene G-BOX Chem XR5, Vacutec (South Africa) and GeneTools analysis software was used to quantify the bands.

2.8 Determination of protein concentration

Protein concentration was estimated using biuret assay (Gornall et al. 1949). The biuret reagent was prepared by first dissolving 0.3 % CuSO₄·5H₂O and 0.9 % KNaC₄H₄O₄·H₂O in 500 ml of 0.2 M NaOH, followed by the addition of 0.5 % of KI and the volume made up to 1 liter with the solvent. Protein standards (concentration 0-10 mg/ml) of bovine serum albumin (BSA) were prepared. 3 ml of the biuret reagent was added to 2 ml final volume of protein.
samples. The resulting solution was mixed and incubated for 10 min at 37 °C in a water bath. The tubes were cooled and the absorbance was read at 540 nm. Readings from the protein standards were plotted against their known protein concentrations (see appendix 2) and the unknown protein concentrations were interpolated from the standard curve.

2.9 Histopathological studies

Histopathological studies was done using the standard laboratory protocol for paraffin embedding. The intestinal segments fixed in 10 % formalin were dehydrated in increasing concentration (50 %-90 %) of ethanol and finally xylene. The tissues were then oriented longitudinally and cross-sectionally and embedded in paraffin wax. 4 µm of sections were cut using a microtome. The slides were allowed to dry overnight and then deparaffinized in p-xylene and rehydrated in gradients of ethanol (100 %, 80 %, 70 %, and 50 %) and rinsed in water. The slides were immediately stained for 5 min in hematoxylin stain and rinsed with water followed by counterstaining in eosin for 3 min. The slides were then mounted in DPX, cover-slipped and allowed to settle and dry for two days before scanning using Leica SCN400, Germany (Software version: SlidePath Gateway Client Viewer 2.0).

2.10 Data analysis

All data were expressed as means ± standard deviation (SD). Statistical analysis was done using Graph Pad InStat Software (version 4.00, Graph Pad Software, San Diego, California, USA). Statistical comparison among groups was done using one-way analysis of variance (ANOVA) followed by Turkey-Kramer multiple comparison test. Value of \( p < 0.05 \) determined using SPSS program was considered to be statistically significant.
CHAPTER THREE

Results

3.1 Type 1 diabetes

3.1.1 Confirmation of diabetes

Fasting (12 hr) blood glucose (FBG) was determined one week after induction with 60 mg/kg body weight STZ for the confirmation of type 1 diabetes. Additional FBG was determined at the end of the two week treatment period. All groups of diabetic rats were above 18 mmol/L throughout the experiment period whereas the non-diabetic groups maintained a normal range of fasting blood glucose as indicated in the methods section.

3.1.2 Change in body weight and small intestine morphometry

3.1.2.1 Body weight and relative small intestine weight

Figure 3.2A shows the body weight change for type 1 diabetic rats and how it is affected by OA treatment compared to non-diabetic groups. One week after induction of type 1 diabetes all diabetic rat groups lost 6-9 % of their baseline weight. On the other hand, the non-diabetic groups gained 7-9 % of their initial weights. However, after two weeks of treatment, both diabetic and non-diabetic groups gained weight. Weight gained is lower (3-11 %) in all diabetic groups compared to the non-diabetic groups (15-27 %). OA treatment of non-diabetic rats led to higher weight gain (27 %) among all groups while insulin treatment of diabetic rats significantly (P<0.05) ameliorated weight loss.

The weight of the small intestine expressed per gram body weight (BW) was increased significantly (p<0.05) in all type 1 diabetic groups compared to the NC group (Figure 3.2B). Moreover, all non-diabetic drug-treated groups (NOI, NI, NOI) also showed increased
intestinal weight expressed per g BW compared to NC group although not statistically significant (p>0.05). The value of 0.0352 g/g BW obtained for NO group is relatively higher than those obtained for NI (0.03415 g/g) and NOI (0.0323 g/g) groups. Furthermore, the weight of SI expressed per unit length was relatively similar in both the diabetic and non-diabetic groups when expressed per g BW (Figure 3.2B). The circumferential area (Figure 3.1) of the intestine from diabetic rats was also increased significantly in all segments compared to the non-diabetic groups as shown in Figure 3.1C. Treatment with insulin or insulin + OA tended to have some ameliorative effects in the duodenum (not significant) and ileum (significant for the DOI group) but not in the jejunum.

**Figure 3.1:** H&E stain of SI showing elongated villi in DC jejunum (A) compared to NC jejunum (B). (C) Cross-section of SI. Magnification (5 ×500 µm)
Figure 3.2: Effects of diabetes and treatments on body weight and SI weight (A) Body weight change (B) Relative SI weight per g body weight/per unit length (C) SI circumferential area. Results are expressed as means ± SD (n = 5-6). Different alphabets (a-c) on a chart indicates significant (p<0.05) difference (Turkey’s range post hoc test).
3.1.2.2 SI morphometry

The average villus length and surface area increases from duodenum and jejunum down to the ileum (Figure 3.3A). However this trend was altered in some groups, most noticeably in the non-diabetic group treated with OA (NO group). In this group, the villi were significantly (p<0.05) longer and broader in the jejunum than the duodenum and ileum (Figure 3.3B). Moreover, this increase in the surface area of the jejunal villi was almost 300 % compared to the NC group. Furthermore, in terms of surface area all diabetic groups also have significantly larger villi in the duodenum and jejunum except for the duodenum of DOI group which was within NC range as shown in Figure 3.3B. The villi in the ileum of all groups were not significantly affected except for NOI group which appeared broader.

Average mucosal height (average villus height + average crypt depth) was visually (Figure 3.1) and significantly increased in the duodenum and jejunum of diabetic rats as well as NO and NOI groups compared to the NC group. The average crypt depth was not adversely altered in all SI segments among groups, hence the significant increase in mucosal height in diabetic groups and NO/NOI groups are largely a result of increased villi length (Figure 3.4A-C).
Figure 3.3: Effects of diabetes and treatments on villi appearance (A) Pattern of villi length along the segments of the SI (B) Average villus surface area in the SI segments. Results are expressed as means ± SD (n = 5-6). Different alphabets (a-d) on a chart indicates significant (p<0.05) difference (Turkey’s range post hoc test).
Figure 3.4: Effect of diabetes and treatments on average crypt depth, average villi length and average mucosal height in (A) Duodenum (B) Jejunum (C) Ileum. Results are expressed as means ± SD (n = 5-6). Different alphabets (a-d) on a chart indicates significant (p<0.05) difference (Turkey’s range post hoc test).
The change in total muscular thickness (comprising the layers from muscularis externae to the serosal side) is presented in Figure 3.5. The change in thickness was only significant (p<0.05) in the DC group (jejunum and ileum) and DO group (jejunum) compared to the NC group.

**Figure 3.5:** Effects of diabetes and OA treatment on muscular thickness. Results are expressed as means ± SD (n = 5-6). Different alphabets (a-b) on a chart indicates significant (p<0.05) difference (Turkey’s range *post hoc* test).

Table 3.1 shows the number of goblet cells (GC) per villus in the SI segments of each of the groups. Diabetes resulted in significant (p<0.05) increase in number of GC per villus. This increase was significantly (p<0.05) reduced in the duodenum and ileum of diabetic rats treated with insulin and in the jejunum and ileum the DOI group. Insulin treatment of normal rats resulted in elevated number of GC per villus in all segments of the SI and this is significant (p<0.05) in the duodenum.
Table 3.1: Effects of diabetes and treatments on SI goblet cells

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<th>Goblet cells per villus</th>
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<td>12±3(^b)</td>
<td>8±2(^b)</td>
<td>10±1(^b)</td>
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Results are expressed as means ± SD (n = 5-6). Different alphabets (a-b) along a row indicates significant (p<0.05) difference (Turkey’s range post hoc test).

3.1.3  Enzymes activity and glutaminase expression

Diabetic groups and non-diabetic treated groups tended to have slight but significant (p<0.05) decrease in phosphate dependent glutaminase (PDG) activity compared to the NC group (Figure 3.6A). The expression of PDG in all groups is not altered to an extent detectable by dot blot analysis (Figure 3.6B). The initial attempt was to quantify the expression using western blot as there was good protein distribution in the samples (appendix 3). However, an unidentified problem that was most likely related to the new blotting paper acquired by the laboratory did not allow successful western blot. The problem is still being investigated with the supplier. Off-cut strips from the old blotting paper were used for the dot blots and the result in Figure 3.6C (and Figure 3.13C) was obtained.
Figure 3.6: Effect of diabetes and treatments on PDG activity and expression. (A) PDG activity. Results are expressed as means ± SD (n = 4). The alphabet (a) on the chart indicates significant (p<0.05) difference (Turkey’s range post hoc test) from other groups. (B) Dot blots (15 µg protein was loaded for each dot). The product of a house-keeping gene β-Actin acted as the loading control. (C) PDG expression normalized with β-Actin.

On the other hand, the SI activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were both significantly elevated in the DC compared to the NC group (Figure 3.7A). The activity of ALT was significantly (p<0.05) decreased in the DO, DI and DOI groups compared to the DC group. Moreover, the treatments in the diabetic groups were able to reduce AST activity and this decrease was significant (p<0.05) except for the DO group. Treatment of normal rats with insulin alone tends to relatively reduce both ALT and
AST activity compared to the NC group and this is significant (p<0.05) in the case of ALT. On the other hand, SI glutamate dehydrogenase (GDH) activity was elevated more than 100% in all diabetic groups and this is significantly more profound in the DO group compared to the DC group (Figure 3.7B).

**Figure 3.7:** Effect of diabetes and treatments on SI enzymes activity (A) ALT and AST (B) GDH. Results are expressed as means ± SD (n = 4). Different alphabets (a-c) on a chart indicates significant (p<0.05) difference (Turkey’s range post hoc test).
The activity of hexokinase (HK) was slightly decreased in the NI, NOI and DI groups and slightly increased in the DC and DO groups although not statistically significant compared to the NC group (Figure 3.8). However there was a significant increase in the DOI group which compared to DC group.

**Figure 3.8**: Effect of diabetes and treatments on hexokinase activity. Results are expressed as means ± SD (n = 4). Different alphabets (a-d) on a chart indicates significant (p<0.05) difference (Turkey’s range *post hoc* test).
3.2 Type 2 diabetes

3.2.1 Confirmation of diabetes

Fasting (12 hr) blood glucose was determined one week after induction with 45 mg/kg body weight STZ for the confirmation of type 2 diabetes. Additional FBG was determined after two weeks of the treatment period. All groups of diabetic rats were within 7-14 mmol/L throughout the experiment period whereas the non-diabetic groups maintained a normal range of fasting blood glucose as indicated in the methods section.

3.2.2 Change in body weight and small intestine morphometry

3.2.2.1 Body weight and relative small intestine weight

Data on changes in body weights one week after induction and after treatment for type 2 diabetic rats is presented in Figure 3.9A. All groups gained weight 1 week after induction but the weight gain in non-diabetic groups is higher than in diabetic groups. At the end of the two weeks treatment, the NC and NO groups gained 10 % and 19% respectively of while the DC and DO diabetic groups gained 0-4 % of their initial weights. There was a slight weight loss in the DM group.

The relative SI weight expressed per BW or unit length for the type 2 diabetic rats is presented in Figure 3.9B. Both parameters were increased significantly (p<0.05) in all diabetic groups and insignificantly (p>0.05) in the NO group compared to the NC group. Moreover, the circumferential area of the SI of NO and diabetic groups is increased most notably around the jejunum as depicted in Figure 3.9C. The jejunum appeared significantly (p<0.05) wider in all diabetic groups compared to NC. The width of the duodenum is increased significantly (p<0.05) only in the DC and DM groups while the ileum is significantly (p<0.05) wider in the DM group only.
Figure 3.9: Effects of diabetes and treatments on body weight and SI weight (A) body weight change (B) Relative SI weight per g body weight/per unit length (C) SI circumferential area. Results are expressed as means ± SD (n = 5-6). Different alphabets (a-c) on a chart indicates significant (p<0.05) difference (Turkey’s range post hoc test).
3.2.2.2 SI morphometry:

The average villus length increases from duodenum through the jejunum down to the ileum (Figure 3.10A). This pattern was not affected in all diabetic groups but OA treatment of normal rats grossly increased jejunal villi length and significantly increased surface area as well (Figure 3.10B). Moreover, the average villus surface area in all segments was not altered in the DC and DO groups but was significantly (p<0.05) increased in the DM group.

Average mucosal height (average villus height + average crypt depth) was not affected in the duodenum and ileum of both DC and DO groups (Figure 3.11 A&C). However, the mucosa is significantly (p<0.05) longer in the jejunum of DC compared to NC (Figure 3.11B). This was reduced by OA treatment of diabetic animals. Mucosal height is significantly higher (p<0.05) in all SI segments of DM group compared to NC. Moreover, OA treatment of non-diabetic rats (NO groups) resulted in significantly longer duodenal and jejunal mucosa compared to NC group. The average crypt depth was slightly decreased in the duodenum of DC and DO groups and ileum of DO groups compared to NC. Furthermore slight elevation of crypts was observed in jejunum of NO and DM groups as well as the ileum of DM group compared to NC. However, in all cases of significant mucosal height change, increase villi length is responsible for at least 80 % of the increase.

Table 3.2 shows the number of goblet cells per villus in the SI segments of each of the groups. Goblet cells per villus were significantly (p<0.05) elevated in all segments in the DM group only compared to other groups.
**Figure 3.10:** Effects of diabetes and treatments on villi appearance (A) Pattern of villi length along the segments of the SI (B) Average villus surface area in the SI segments. Results are expressed as means ± SD (n = 5-6). Different alphabets (a-b) on a chart indicates significant (p<0.05) difference (Turkey’s range post hoc test).

**Table 3.2:** Effects of diabetes and treatments on SI goblet cells

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<thead>
<tr>
<th></th>
<th>DUODENUM</th>
<th>JEJUNUM</th>
<th>ILEUM</th>
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<td></td>
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<td>NO</td>
<td>DC</td>
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<td>Goblet cells per villus</td>
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<td>4±2&lt;sup&gt;a&lt;/sup&gt;</td>
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Results are expressed as means ± SD (n = 5-6). Different alphabets (a-b) along a row indicates significant (p<0.05) difference (Turkey’s range post hoc test).
Figure 3.11: Effect of diabetes and treatments on average crypt depth, average villi length and average mucosal height in (A) Duodenum (B) Jejunum (C) Ileum. Results are expressed...
as means ± SD (n = 5-6). Different alphabets (a-c) on a chart indicates significant (p<0.05) difference (Turkey’s range post hoc test).

The change in total muscular thickness (comprising the layers from muscularis externae to the serosal side) is presented in Figure 3.12. The change in thickness was only significant (p<0.05) in the duodenum of DM group, jejunum of NO and DO groups and ileum of DC and DM groups compared to NC group.

![Figure 3.12: Effects of diabetes and treatments on muscular thickness. Results are expressed as means ± SD (n = 5-6). Different alphabets (a-c) on a chart indicates significant (p<0.05) difference (Turkey’s range post hoc test).](image)

### 3.2.3 Enzymes activity and glutaminase expression

Glutaminase activity was slightly decreased in the DC group but this decrease was insignificant (p>0.05) compared to the NC group as shown in Figure 3.13A. The expression of PDG was also not different to a large extent as detected by dot blots (Figure 3.13B). Treatment with OA of both non-diabetic and diabetic groups significantly (p<0.05) decreased PDG activity while DM group have a normal PDG activity.
Figure 3.13: Effect of diabetes and treatments on PDG activity and expression. (A) PDG activity. Results are expressed as means ± SD (n = 4). Different alphabets (a-c) on a chart indicates significant (p<0.05) difference (Turkey’s range post hoc test). (C) Dot blots (15 µg protein was loaded for each dot). The loading control was β-actin. (C) PDG expression normalized with β-actin.

On the other hand, diabetes caused a significant increase in ALT and AST activity and this increase was reduced by treatment with OA or metformin particularly with respect to ALT whose levels were reduced to those found in the normal animals (Figure 3.14A). GDH activity was also significantly increased by diabetes and this increase was reduced by treatment with OA or metformin, the reduction being significant with the latter treatment (Figure 3.14B).
Figure 3.14: Effect of diabetes and treatments on SI enzymes activity (A) ALT and AST (B) GDH. Results are expressed as means ± SD (n = 4). Different alphabets (a-c) on a chart indicates significant (p<0.05) difference (Turkey’s range post hoc test).

The SI hexokinase activity was decreased in the DC group although this was not significant compared to NC group (Figure 3.15). Moreover, OA treatment in both NO and DO group did not significantly affect HK activity although there was a slight increase in activity in the DO group compared to NC. The HK activity in the DM group was significantly (p<0.05) higher compared to the DC group.
Figure 3.15: Effect of diabetes and treatments on hexokinase activity. Results are expressed as means ± SD (n = 4). Different alphabets (a-b) on a chart indicates significant (p<0.05) difference (Turkey’s range post hoc test).
CHAPTER FOUR

Discussion

4.1 Type 1 Diabetes

Prevention of weight loss by treatment of diabetic rats with oleanolic acid alone and in combination with insulin has been reported in a 5 weeks study on STZ induced diabetic-rats (Musabayane et al., 2010). In the present study, both NO and NOI groups gained relatively higher weight compared to NC (Figure 3.1A) which may suggest OA-induced tissue growth even in the absence of diabetes. Because OA has not been previously reported to induce increased food consumption or water intake in various rat models (Musabayane et al., 2010; Raphael and Khuttan, 2003; Wang et al., 2010), increased nutrient consumption could not explain the weight gain. Hence, OA may induce tissue growth via a different mechanism such as increased delivery of energy source into the cells. The short duration of the present study did not allow for optimum time to observe increased weight gain in diabetic animals as reported by Musabayane et al. (2010).

Increased weight and length of the small intestine have been noticed in other studies on animal models of diabetes (Diani et al., 1976; Schedl and Wilson, 1971). This has been found to be both a result of hyperplasia and hypertrophy of the SI (Nakabou et al. 1974). In this study, all treatments were not able to prevent increase in both parameters (weight and length) (Figure 3.1B). This could be due to the fact that increased intestinal weight and length occur in the early stages of chemical-induced diabetes. Significant SI weight and length increases were reported to occur as early as 8 days post diabetes induction (Schedl and Wilson, 1971). Hence the one week window period given for the confirmation of diabetes may have induced an intestinal growth effect that cannot be reversed in the two weeks treatment period. The
significant reduction observed on the increased circumferential area in the ileum of DOI group and slight decrease in duodenum and ileum of DI group suggest that SI segments respond to treatment differently due to their metabolic specialization. This could be attributable to the absorptive nature of the jejunum since hyperphagia induced by diabetes has been suggested to be a trigger of intestinal growth (Thulesen et al. 1999). It is important to mention at this point that hyperphagia is just one of a number of explanations given for increased SI growth none of which is yet conclusive (Zhao et al. 2003). These include a glucagon like peptide-2 (GLP-2) -which possesses SI trophic effects- mediated mechanism.

In line with this theory, GLP-2 level has been observed to be elevated in diabetic condition (Fischer et al., 1997). Hence multiple factors are likely to be responsible for the observed effects of diabetes on the SI.

The morphometric data also seem to suggest the proximal SI (jejunum and the duodenum) to be the main site of diabetes induced SI changes as observed in the significant increase in mucosal and villi length and surface area in all diabetic groups around the jejunum (Figure 3.4A-C). The villi in the ileum are not affected by either treatments or diabetes and this could be attributed to its minimal or no function in nutrient absorption. On the other hand, surprisingly profound increase in length and surface area of jejunal villi of normal rats with OA treatment is reported here for the first time. Increased food as well as increased glucose (and other nutrients) absorption as given in the explanation for diabetes-induced mucosal changes could not however account for the OA-induced villi changes in normal rats for the following reasons: 1) as stated earlier, OA has not been reported to increase nutrient uptake 2) although OA induces gross villi changes, the whole intestinal weight or length was only slightly affected by OA treatment suggesting minimal hypertrophy. These suggest an entirely different mechanism of OA-induced villi changes in the SI. Suppression of apoptosis in a caspase 3-related mechanism has also been suggested for diabetes induced SI changes (Noda
et al., 2001). However, various reports suggest that OA and other pentacyclic triterpenes do not affect caspase-3 in intestine IEC-6 and IEC-18 cell lines (Reyes et al. 2006) as well as liver L-02 cell lines (Yan et al., 2010). Hence, mechanisms that have been investigated for SI hypertrophy and villi changes could not account for OA induced changes in the SI of normal rats. Another putative mechanism that has not been yet investigated is the inhibitory effects of OA (Takada et al., 2010) on the apoptotic tumor necrosis factor-α (TNF-α) which is known to induce small intestine damage (Fukumoto et al., 2011, Matsumoto et al., 2006). If OA does inhibit TNF-α in the mucosal cells to any significant extent, it will be expected to induce mucosal hyperplasia as observed in the present study.

The actual causes of increased muscular stiffness induced by diabetes have not been yet elucidated conclusively. However, orientation and content of collagen of the SI wall (Fung, 1993) as well as collagen glycation (Kandemir et al., 1995) have been implicated for the effect. In another study, increased advanced glycation end products (AGEs) and receptors for advanced glycation end products (RAGEs) were detected in type 1 diabetic rats (Chen et al., 2012). Hence, the observed significant decrease in muscular thickness in all diabetic treated groups may be related to the decreased circulating glucose with minimal collagen glycation that occurred with the treatments.

Diabetes has been reported to increase the number of goblet cells per villus (Mantle et al., 1989) as confirmed by this study. Increased in number of goblet cells as well as mucin secretion has been reported to be associated with many gastrointestinal diseases such as zoonotic infections (Kim and Khan, 2013), carcinoma and inflammatory bowel disease (Filipe et al., 1979, Reid et al., 1984). However, diabetes appeared to affect only the number of goblet cells per villus but mucin content per tissue was decreased (Mantle et al., 1989). Therefore, diabetes affects goblet cells function differently from other GI diseases. Although no conclusion can be drawn on the actual cause of increased number of goblet cells, factors
such as hyperphagia that cause alterations in villi size have been implicated (Mantle et al., 1989). However, in the present study, OA treatment of normal animals has also resulted in larger villi but the number of goblet cell per villus is not different from the normal control. This is not surprising since OA does not lead to hyperphagia in normal animals and hence the non-elevation in number of goblet cells per villus in the NO group. Moreover, the rise in number of GC per villus has not been attributed to hyperglycaemia and this study also seems to support this assertion. The significant rise in GC number per villus in normal rats treated with insulin suggest a mechanism other than hyperglycaemia (and increase in villi size probably) for elevated GC. However the implication of increased villus size is depicted in the DC, DO and DOI which have high GC per villus as well as larger villi compared to the DI group which has lower of both (Table 3.1). But even this is refuted by the above mentioned observation with NO group. Hence, more studies to elucidate the actual cause in alteration in number of GC per villus in relation to glucose level and of course diabetes are therefore needed for conclusive information.

The activity of PDG could be increased greatly under prolonged fasting and uncontrolled diabetes in accordance with its role in SI gluconeogenesis under these conditions (Mithieux et al., 2004). However, slight decrease to no effect was observed in early stages of fasting and diabetes as reported in this study (Hartmann and Plauth, 1989; Nagy and Kretchmer, 1986). The rate limiting step for PDG catalyzed reaction was suggested to be the deamidation step and not the removal of the product (glutamate) (Hartmann and Plauth, 1989). In another study a normal mitochondrial PDG from SI was able to generate glutamate in a concentration more than what can be handled by the aminotransferases (ALT and AST) responsible for the greater part of its subsequent metabolism (Masola et al., 1985). These imply that, increased glutamate utilization can occur without affecting PDG activity under different metabolic states. Essentially this indicates that increased aminotransferase activity may occur as a result
of conditions such as diabetes without significant alteration in PDG activity. Furthermore, the observed decrease in diabetes-induced increased ALT and AST activities in the DO and DOI groups as well as slight increase in HK activity in the DOI group is not directly explainable in terms of OA’s ability to induce glucose removal from the circulation as in the case of insulin (Figure 3.6 A&C). The insulin effects on HK may be explained by the increased utilization of glucose in other tissues making the intestine less important as site of glucose disposal. Consequently, reduced cytosolic pyruvate from glycolysis for transamination with available glutamate explains decreased ALT activity with insulin treatment. However, in the case of OA treatment, disparity was observed in HK activity in relation to ALT and AST activities. Increase in HK activity was maintained or even increased (in DOI group) with OA treatment while increased ALT and AST activities were reduced. The implication is that, some of the glucose-6-phosphate generated by HK is being committed to other pathways (glycogen synthesis or pentose phosphate pathway). Hence, oleanolic acid treatment maintains the importance of small intestine as a site of excess glucose disposal (even in the presence of insulin) while decreasing diabetes-induced elevated ALT and AST activities. This observation further confirms a synergistic relationship between OA and insulin in alleviating diabetes.

Flux through intestinal glutamate dehydrogenase is known to be minimal and less important than the aminotransferases pathway for glutamate utilization (Newsholme and Carrie, 1994). However, under conditions such as starvation, generation of ammonia from glutamate in the intestine has been reported to be increased as an adaptive mechanism for modulating redox potential of the whole body (Souba, 1993). Hence, the GDH pathway could also be increased (and important) in diabetes to generate more ammonia for redox control of diabetes induced ketoacidosis. The α-ketoglutarate generated by the reaction can be converted to oxaloacetate and be available for further transamination with glutamate which further explains the
increased AST activity observed in this study. However, the data available is not sufficient to confirm that increase in GDH activity in the SI during diabetes is an adaptive response for controlling diabetic ketoacidosis. If it happens to be so, the further increase in GDH activity in the DO group may hence suggests further beneficial effects of OA in modulating diabetes induced ketoacidosis.

4.2 Type 2 Diabetes

As noted earlier, the synergestic effect of OA and insulin in type 1 diabetic rats has been reported elsewhere (Musabayane et al. 2010). Since there is residual insulin in the type 2 diabetic model used in the study, the observed effect of OA will be in synergism with insulin. In the first week after induction of diabetes the weight of the diabetic animals did not change significantly from that of control. However, after the 2 weeks of treatment the change in body in diabetic animals was negligible suggesting a less severe diabetes than what was observed with type 1 diabetes.

Most of the previous studies have reported SI changes in type 1 model of diabetes (Diani et al., 1976; Schedl and Wilson, 1971). It is confirmed here that type 2 model of diabetes also impart the same effect in terms of increase SI weight and length. Treatment of type 2 diabetic rats with both OA and metformin resulted in relatively higher SI weight/body weight as well as SI weight/unit length (Figure 3.7B). This effect could be attributed to the effect of both compounds on the absorptive and metabolic properties of the SI. Oleanolic acid has been reported to decreased glucose absorption and increased glycogen synthesis in an isolated SI from non-diabetic animals (Khathi et al., 2013). Similarly, metformin has been reported to decrease absorption and increase utilization of glucose by the small intestine (Bailey et al., 1994). All of these effects could potentially induce intestine-trophic effects via adaptive mechanisms that can involve the incretins as well as cytokines (see discussion on type 1).
From Figure 3.7C, the duodenum and jejunum appear to be the main sites for the changes on circumferential area except for the metformin treated group which was seen to impact changes in all intestinal segments.

Type 2 diabetes appeared to cause minimal changes in the villi morphology as shown in Figure 3.8B and Figure 3.9A-C as well as muscular stiffness (Figure 3.10) and number of goblet cells (Table 3.2). This could be due to the lower hyperglycaemia in type 2 diabetic model compared to the other reports on type 1 diabetes. This further suggests a causative relationship between high blood glucose and SI morphometric changes.

Both the specific activities of PDG and HK were not affected by type 2 diabetes suggesting unaltered roles of these metabolites in a less severe form of diabetes. The slight increase in HK activity in diabetic rats by metformin and OA treatments suggests induced increase SI glucose utilization. However, the decreased ALT and AST activities with same treatments indicate that the generated glucose-6-phosphate was not used for pyruvate generation but rather used for other pathways such as glycogen synthesis (Kathi et al., 2013, Bailey et al., 1994).

4.3 Summary

Blood glucose level (associated with type 1 and type 2 models), duration of diabetes and delay of treatment appear to play important roles in aggravating the extent of diabetes-induced morphological changes. Type 1 diabetes (or higher blood glucose) induced more severe changes in terms of small intestine weight and length, villi size, underlying muscle thickness and goblet cells per villus than type 2 diabetes (or lower blood glucose). This was also true for ALT, AST and GDH activities which showed elevation due to diabetes while PDG was only slightly affected. The activities of ALT, AST and GDH were reversed by OA treatment in both studies. Both type 1 and type 2 diabetes may hence induce morphological
changes as well as changes in upstream enzymes of glutamine metabolism without largely affecting PDG. Moreover, because the small intestine is the site of absorption of orally delivered drugs such as OA and metformin, these drugs also induce additional morphological changes in the SI. This may explain the gastrointestinal disturbances associated with diabetes as well as oral antihyperglycaemic agents (OHA). This study also highlights the significance of treating normal animals with treatment drugs as this can give significant information on the side effects of the drug.
CHAPTER FIVE

Conclusions

General Conclusion

In the early stages of diabetes, the activity of PDG slightly decreases and this is not reversed by OA treatment in both type 1 and type 2 diabetes model. However, OA prevents diabetes-induced increase in the activities of upstream enzymes of glutamine metabolism while increasing glucose utilization by the intestine during diabetes. This may be an additional mechanism via which OA acts as an antihyperglycaemic agent. Furthermore, although a synergistic relationship exists between OA and insulin in alleviating some diabetes induced SI metabolic changes observed in the study, the treatments do not reverse the morphological changes induced in early diabetes. On the other hand, the activity of HK, ALT, AST and GDH where increased in the early stages of diabetes. This may be due to increased utilization of the product of PDG catalyzed reaction (glutamate) occurring with slight changes in PDG activity suggesting that existing glutaminase activity is sufficient to provide for increased utilization of its product.

Further research

1) Investigation of the quantitative aspects of the effects of OA on intestinal glutamine utilization.

2) Expression studies on glutamine metabolizing enzymes investigated in this study as well as similar studies on other related enzymes.

3) The mechanism through which OA induces villi changes without significantly affecting intestine weight and length will give insight in to the mechanism of diabetes (as well as other gastrointestinal diseases) induced gastrointestinal changes.
4) The effects of OA on long term metabolic changes induced by diabetes in the SI should be investigated.

5) Effects of OA (as well as metformin) delivered parenterally will give additional information on the importance of SI as site of drug-stimulated glucose disposal as well as elucidation of the importance of SI-drug interaction in the drug-induced morphological changes.

6) The gastrointestinal effects of OA should be investigated further in diabetes as the drug is already an oral subscription drug for liver diseases in some parts of the world (Sheng and Sun, 2011).
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GLOSSARY (www.thefreedictionary.com definitions)

Acidosis: accumulation of excess acid in the body and the lack of sufficient bicarbonate for effectively buffering.

Cytokines: Chemicals made by the cells that act on other cells to stimulate or inhibit their function.

Adipocytes: a fat (adipose) cell, potentially containing a large fat vacuole consisting mainly of triglycerides.

Adipokines: Autocrine and paracrine factors released from human adipose tissue, in particular the visceral depots; including cytokines, acute phase reactants, growth factors, and other inflammatory mediators.

Advanced glycation end products: proteins or lipids the become glycated after exposure to sugars.

Allograft: surgical transplantation of tissue between two genetically dissimilar individuals of the same species, such as between two humans who are not monozygotic twins.

Apoptosis: A natural process of self-destruction in certain cells that is determined by the genes and can be initiated by a stimulus or by removal of a repressor agent.

Atherosclerosis: a general term for hardening of the blood vessels.

Cardiovascular: pertaining to the heart and blood vessels.

Cerebrovascular disease: general term for a brain dysfunction caused by an abnormality of the cerebral blood supply.

Chylomicrons: the tiny lipoproteins of approximately 2% protein that convey dietary fat throughout the body.

Coenzyme: an organic nonprotein molecule that binds with the protein molecule to form the active enzyme.

Coronary artery disease: Coronary artery disease is a narrowing or blockage of the arteries and vessels that provide oxygen and nutrients to the heart.

Cosubstrate: the second or other substrate of a multisubstrate enzyme.

Deamination: removal of the amino group, —NH2, from a compound.

Depolarisation: to deprive of polarity by uneven ion distribution across a membrane.

Dimer: a compound formed by combination of two identical molecules.

Dyslipidemia: abnormality in, or abnormal amounts of, lipids and lipoproteins in the blood.

Endothelium: the layer of epithelial cells that lines the cavities of the heart, the serous cavities, and the lumina of the blood and lymph vessels.
**Exocytosis:** A process of cellular secretion or excretion in which substances contained in vesicles are discharged from the cell by fusion of the vesicular membrane with the outer cell membrane.

**Gastric:** pertaining to, affecting or originating in the stomach.

**Glocosuria:** Excretion of glucose in the urine, especially in elevated quantities.

**Glycoconjugates:** carbohydrates covalently linked with other chemical species such as proteins, peptides, lipids and saccharides.

**Vagal:** relating to the vagus nerve (the tenth of twelve (excluding CN0) paired cranial nerves).

**Hormone:** a chemical released by a cell, a gland, or an organ in one part of the body that affects cells in other parts of the organism.

**Hyperphagia:** a medical sign meaning excessive hunger and abnormally large intake of solids by mouth.

**Hypertension:** a chronic medical condition in which the blood pressure in the arteries is elevated.

**Hypertrophy:** increase in the volume of an organ or tissue due to the enlargement of its component cells.

**Mesangial cells:** specialized cells around blood vessels in the kidneys, at the mesangium.

**Myocardiac infarction:** the medical term for an event commonly known as a heart attack.

**Nephropathy:** A disease or abnormality of the kidney.

**Neuropathy:** A disease or abnormality of the nervous system.

**Oxidative stress:** A condition of increased oxidant production in animal cells characterized by the release of free radicals and resulting in cellular degeneration.

**Peripheral vascular disease:** refers to the obstruction of large arteries not within the coronary, aortic arch vasculature, or brain.

**Postprandial:** following a meal.

**Proteinuria:** the presence of excessive amounts of protein in the urine.

**Retinopathy:** a pathological disorder of the retina.

**Sepsis:** the presence of pathogenic organisms or their toxins in the blood or tissues.
APPENDIX 1

Ethics approval for the study

21 October 2013

Reference: 001/14/Animal

Mr M Isah
Biochemistry
School of Life Sciences
University of KwaZulu-Natal
WESTVILLE Campus

Dear Mr Isah

Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2013/2014 on the following project:

"Effects of orally delivered oleanolic acid on glutamine metabolising enzymes in the small intestine of type 1 and type 2 diabetic rats."

Yours sincerely

[Signature]
Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc  Registrar – Prof. J Meyerowitz
     Research Office – Dr N Singh
     Supervisor – Dr B Masola
     Head of School – Prof. S Mukaratirwa
     BRU – Dr S Singh
APPENDIX 2

Standard curve for the determination of protein concentration using Biuret assay
APPENDIX 3
SDS-PAGE gels of the samples

TYPE 1

Reducing SDS-PAGE (10 %) showing protein distribution at 50 µg protein. Mw = molecular weight marker.

TYPE 2

Western blot showing successful transfer of the molecular weight marker (50 µg loading protein).