UNIVERSITY OF KWAZULU-NATAL

THE EFFECTS OF NARINGIN ON GLUCOSE TOLERANCE AND KETOACIDOSIS IN TYPE 1 DIABETES

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2014

THE EFFECTS OF NARINGIN ON GLUCOSE TOLERANCE AND KETOACIDOSIS IN TYPE 1 DIABETES

BY

MURUNGA ALFRED NEONDO

212552078

Submitted in partial fulfillment of the requirements for the award of the degree of

MASTER OF PHARMACY (PHARMACOLOGY)

Department of Pharmacology

Discipline of Pharmaceutical Sciences

College of Health Sciences

University of KwaZulu-Natal

Supervisor: Dr. P.M.O. Owira

Date of submission: December 2014

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PREFACE

The experimental work described in this dissertation was carried out in the Department of Pharmacology, Discipline of Pharmaceutical Sciences, College of Health Science, University of KwaZulu-Natal, Durban from March 2012 to January 2014 under the supervision of Dr. Owira P.M.O.

The study is an original work of the author and has been submitted in fulfillment of the academic requirements for obtaining a Msc. Degree in Pharmacology. Information from other sources used in this dissertation has been duly acknowledged in the text and reference section.

Murunga Alfred Neondo

Dr. Owira P.M.O (Supervisor)

DECLARATIONS

DECLARATION 1 – PLAGIARISM

I, **Murunga Alfred Neondo** declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) Their words have been re-written but the general information attributed to them has been referenced.
b) Where their exact have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

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DECLARATION 2 – PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

List of Publications

1. Murunga AN, Owira PM

   Diabetic Ketoacidosis: an overlooked child killer in sub-Saharan Africa?

   Trop Med Int Health. Volume 18, Issue 11, pages 1357-1364, November 2013.

ACKNOWLEDGEMENTS

Thanks to God Almighty

My sincere gratitude and appreciation to:

- My parents, Mr. and Mrs. Murunga for their love and support throughout the study.
- My supervisor, Dr. Owira P.M.O for his guidance, support and motivation throughout the study. I admire the spirit, creativity and desire for excellence which always inspired me.
- My colleagues Cobongela S., Nkomo S., Nzuza S., Ngubane S., Hayangah J., Dr. Adebiyi O., Dr. Adebiyi A. and Tlaila, T. for all the accomplishments together. I will forever be grateful.
- Dr. Onyango M. D. for his guidance and support.
- My friends from Physiology Department Simo Zulu, Siyabonga Sibiya.
- The staff at Breeding Research Unit (BRU) for all their support.
- The College of Health Sciences for the financial support granted during my study.

LIST OF ABBREVIATIONS

2,3-DPG	2, 3-Diphosphoglycerate
3HB	3-β-hydroxybutyrate
AcAc	Acetoacetate
AG	Anion gap
AMPK	AMP-activated protein kinase
Apo	Apolipoprotein
CAD	Coronary artery disease
CETP	Cholesterol ester transfer protein
CPT	Carnitine palmitoyl-O-transferase
DAG	Diacyl glycerol
DKA	Diabetic ketoacidosis
DM	Diabetes mellitus
ECG	Electrocardiogram
F-1-6-P	Fructose-1,6-biphosphatase
FBG	Fasting blood gucose
FFA	Free fatty acids

FOXO	Fox head box protein O
G6Pase	Glucose-6-phosphatase
GFR	Glomerular filtration rate
GK	Glucokinase
GLUT	Glucose transporter
GS	Glycogen synthase
GSK3	Glucose kinase 3
HDL	High density lipoprotein
HIV/ AIDS	Human immunodeficiency virus/ Acquired Immunodeficiency disease syndrome
HLA	Human leucocyte antigen
HMG-CoA	Hydroxymethyl glutyryl Coenzyme A
HMGCR	Hydoxymethyl glutyryl CoA reductase
HRP	Horse radish peroxidase
IGT	Impaired glucose tolerance
IL-6	Interleukin factor 6
IM	Intramusucular
IRS	Insulin receptor substrate

IV	Intravenous
LCFA-CoA	Long chain fatty acid-coenzyme A
LDL	Low density lipoprotein
MAT	Methyl acetoacetyl thiolase
NAD	Nicotinamide adenine dinucleotide (Oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
OD	Optical density
PDK1	Phosphoinositide dependent protein kinase 1
PEPCK	Phosphenolpyruvate carboxykinase
PH	Pleckstrin homology
PI3K	Phosphatidylinositol-3-kinase
PK	Pyruvate kinase
PKC	Protein kinase C
SC	Subcutaneous
SCOT	Succinyl CoA-oxoacid transferase
SGLT	Sodium glucose linked transporter
TG	Triglycerides

TNF-α	Tumor necrosis factor-α
VLDL	Very low density lipoprotein

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ABSTRACT

The effects of naringin (4',5,7-trihydroxy flavonone-7-rhamnoglucoside), a flavonoid isolated from the grapefruit and other citrus fruit species were investigated on diabetic ketoacidosis (DKA) in a type 1 diabetic rat model. DKA is an acute life threatening complication of diabetes mellitus. Male Sprague-Dawley rats (225-250g) were divided into 5 groups (n=7). Group 1 (control) was treated daily with 1.0 ml/kg distilled water while group 2 was treated with 50 mg/kg of naringin, via gastric gavage respectively. Diabetes was induced in groups 3, 4 and 5 by a single intraperitoneal injection of 60 mg/kg streptozotocin in 0.1 M citrate buffer and was confirmed after 48 hours. Group 3 was further treated with subcutaneous insulin (4 IU/kg) twice daily respectively. Blood samples for analysis were collected by cardiac puncture. The animals were handled humanely according to the guidelines of the Animal Ethics Committee, University of KwaZulu Natal, number 106/13/Animal.

The untreated diabetic rats (group 5) showed significant ($p<0.0001$) hyperglycemia, polydipsia, polyuria, weight loss, impaired glucose tolerance, low fasting plasma insulin (FPI) and low glycogen levels compared to the normal control. They also showed significantly ($p<0.0001$) elevated acetoacetate (AcAc), β-hydroxybutyrate (3HB), total ketone body (TKB), anion gap (AG) and potassium ($p<0.05$) levels compared to normal control. Furthermore, significant ($p<0.01$) reductions blood pH, sodium, chloride and bicarbonate ($p<0.0001$) levels were recorded compared to the normal control. Treatment of the diabetic groups with naringin did not improve fasting blood glucose and serum electrolyte levels but significantly improved weight loss ($p<0.0001$), water consumption ($p<0.0001$), hepatic glycogen level ($p<0.05$), 3HB ($p<0.05$), AcAc ($p<0.05$), TKB ($p<0.01$), bicarbonate ($p<0.01$), blood pH ($p<0.01$) and AG ($p<0.05$) compared to the diabetic control group.

The results in this study therefore suggest that naringin reverses ketoacidosis but does not improve glucose tolerance in a diabetes type 1 rat model.

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Chapter One

1.0 Introduction

1.1 Background on Diabetes mellitus (DM)

Diabetes Mellitus (DM) is a global health problem with major public health, social and economic implications. It affects millions of people around the world. The International Diabetes Federation (IDF) global estimates indicate that the number of diabetics will increase from 382 million recorded in 2013 to about 592 million by 2035 [1]. The greatest increases in prevalence are expected to occur in developing countries [2] especially in Africa where the number is expected to rise from about 19.8 million in 2013 to about 41.4 million by 2035 [1]. It is currently estimated that out of these numbers, 2.6 million are in South Africa, excluding undiagnosed cases estimated to be 63%) [1]. Thus, the true incidence and impact of diabetes in the region remains largely unknown as the available data could be an underestimation. As a result of the increasing prevalence and incidence of diabetes, a rise in complications leading to increased morbidity and mortality is expected. These include dysfunction, damage and failure of organs and systems such as eyes, nerves, kidney, blood vessels and heart. Eventually, this will have devastating social and economic effects in the region as the most productive members of the society; mostly breadwinners [3] are affected.

1.1.1 Definition and classification of diabetes mellitus

Diabetes mellitus (DM) is defined as a metabolic disorder of multiple aetiology characterised by chronic hyperglycaemia resulting from disturbances in carbohydrate, fat and protein metabolism [4] resulting from defects in insulin secretion, insulin action, or both [5]. It presents with classic

features such as polyuria (frequent urination), polydipsia (frequent thirst) and polyphagia (frequent hunger). It is classified into 2 major types, type 1 and type 2 DM.

Type 1 DM results from the autoimmune destruction of the insulin producing pancreatic beta (β) islet cells [6-8]. The auto antibodies progressively infiltrate the β-islet cells leading to an auto immune cellular mediated destruction. Consequently, the damaged β-cells cannot produce insulin and an absolute deficiency ensues. As a result, lifelong insulin replacement therapy cannot be circumvented. Recent statistics indicate that about 5-10% of people with diabetes have type 1 DM [8] Also referred to as juvenile diabetes, type 1 DM mostly affects children and young adults generally occurring before the age of 40 years [9]. Studies have linked genetic predisposition to the development of type 1 DM with key predisposing factors being human leukocyte antigen (HLA) haplotypes DR4-DQ8 and DR3-DQ2 frequently encountered in children [7] while the protective HLA DR15-DQ6 haplotype, is a rare combination [10]. However, environmental factors have also been identified as possible trigger factors [11].

Type 2 DM accounts for approximately 90-95% of the patients with diabetes [8]. In Africa too, specifically in the Sub-Saharan region, it is also the most common with trends showing increases in prevalence [3]. Among factors attributed to this rapid rise in prevalence include acquired sedentary lifestyles, dietary changes, population ageing, urbanization, poverty and the double burden of communicable diseases like HIV/ AIDS [3], tuberculosis and malaria especially in Sub Saharan Africa [12], a region ravaged by poverty with little to spare for healthcare funding. Type 2 DM occurs as a result of insulin resistance and abnormalities in insulin secretion [13]. Insulin resistance is the inability of target tissues to increase glucose uptake in response to increased insulin levels. Chronic hyperglycemia in the β-cell results in increased sensitivity to glucose, increased basal insulin release, reduced response to stimulus to secrete insulin, and a gradual

depletion of insulin stores [14]. A majority of the people with type 2 DM are obese, with central visceral adiposity a key feature of the metabolic syndrome also characterized by atherogenic dyslipidemia, a pro-thrombotic state, a pro-inflammatory state and hypertension [14, 15].

1.1.2 Regulation of blood glucose homeostasis

Insulin, a polypeptide hormone [16, 17] is secreted by the endocrine pancreatic beta (β) islet cells of Langerhans [18] and is involved in the regulation of glucose homeostasis. The pancreatic islet of Langerhans is also made up of the alpha (α), delta (δ) and pancreatic polypeptide (PP) cells which secrete glucagon, somatostatin and pancreatic polypeptide hormones, respectively [18]. Insulin is secreted primarily in response to glucose although other nutrients such as free fatty acids (FFA) and amino acids can augment its secretion [17]. Its main role is to maintain glucose homeostasis by stimulating the uptake, utilization and storage of glucose in muscle and adipose tissue while inhibiting hepatic production.

The cellular uptake of glucose is mediated by transporters as the lipid bi-layers are impermeable to the carbohydrates. These include a family of facilitative glucose transporters (GLUT) which facilitate a uni-directional and energy independent process and a sodium glucose linked transporter (SGLT) which is an energy dependent process. The SGLTs are mainly limited to the kidney, skeletal muscle and intestines while the GLUTs are widely distributed but exhibit differences in substrate and tissue specificity. Among the GLUT isoforms identified include GLUT 1-4 (Table 1.1).

Glucose transporter	Tissue distribution
Glut 1	Ubiquitous distribution in tissues and culture cells.
Glut 2	Liver, pancreatic β-islets, kidney, small intestine.
Glut 3	Brain and nerve cells.
Glut 4	Insulin responsive tissues like adipose, skeletal and heart muscles.

Table 1.1 Isoforms of glucose transporter molecules (GLUT) [19].

However, of all these GLUTs, only isoform 4 exhibits insulin sensitivity. GLUT-4, a trans-membrane protein is exclusively expressed in the peripheral insulin sensitive tissues, fat and muscle tissues [20]. In the absence of insulin, it is sequestered in intracellular vesicles but on stimulation, it is translocated to the plasma membrane where it mediates glucose uptake [20]. The rest translocate glucose in an insulin independent manner.

Apart from its direct effects on glucose entry, insulin also controls hepatic glucose production by suppressing hepatic glucose synthesis (gluconeogenesis) and stimulating glucose metabolism (glycolysis). This occurs through enhanced gene expression of glycolytic enzymes glucose kinase (GK), pyruvate kinase (PK) while decreasing expression of gluconeogenic enzymes glucose-6-phosphatase (G6Pase), fructose-1,6-biphosphatase (F-1,6-BPase) and phosphoenolpyruvate carboxykinase (PEPCK) [21].

1.1.3 Cellular insulin signaling

Insulin binds to its receptor which is made up of 2 extracellular α-subunits and two trans-membrane β-subunits with tyrosine kinase activity [22, 23]. Insulin binds to the α-subunit and activates the tyrosine kinase in the β-subunit which then auto-phosphorylates intracellular substrates such as the insulin receptor substrate (IRS), specifically isoform 1 (IRS-1) [24]. Upon phosphorylation, the IRS-1 protein interacts with a series of effector molecules containing Src homology 2 (SH2) domains that specifically recognize different phosphotyrosine motifs [22]. These include phosphatidylinositol-3-kinase (PI3K), made up of the p85 regulatory subunit and a p110 catalytic subunit whose activation occurs in response to interaction with the IRS-1 proteins [24, 25]. The activated PI3K catalyzes the change of phosphatidylinositol-4, 5-diphosphate (PIP2) into phosphatidylinositol-3,4,5-triphosphate (PIP3) [24]. PIP3 then activates proteins which contain a pleckstrin homology (PH) domain [22] such as the serine/threonine (Ser/Thr) phosphoinositide dependent protein kinase 1 (PDK1), which phosphorylates and activates several downstream kinases such as Akt/protein kinase B [26]. Akt is involved in stimulating the translocation of a glucose transporter-4 (GLUT-4) containing vesicles from the intracellular to the plasma membrane for glucose uptake [24]. Akt also stimulates the phosphorylation of a variety of proteins that affect cellular metabolism such as glucose kinase-3 (GSK3) [26], normally inactivated following phosphorylation. A key GSK3 substrate is glycogen synthase (GS), the rate limiting step in glycogenesis which catalyses the change of uridine di-phosphate glucose (UDP-glucose) to glycogen [27]. Phosphorylated GS is inactive [28] and this inhibits glycogenesis. Therefore, by Akt phosphorylating and inactivating GSK3 it promotes glycogenesis. Akt also phosphorylates and activates the Forkhead Box Protein O (FOXO) transcription family proteins which results in their localization in the cytoplasm away from their

nuclear targets [24] thus reducing transcription activity. However, in low glucose levels, FOXO localizes in the nucleus and stimulates gluconeogenesis by promoting the expression of key gluconeogenic enzymes glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) [29] (Figure 1.1).

1.1.3.1 Insulin signaling defects in diabetes

Insulin signaling defects have been implicated in the pathogenesis of DM. This is especially the case in type 2 DM although the exact mechanisms are still not well understood. Elevated fatty acids commonly seen in obesity are believed to play a key role. In obesity, adipocytes derived from pleuripotent mesenchymal stem cell precursors undergo low proliferation and differentiation leading to hypertrophic fat cells [30]. This activates the stress and inflammatory pathways [14, 16] which induces its endocrine functions by releasing adipocytokines (adipocyte-secreted proteins) such as the tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), resistin aptly described by Housa *et. al.,* [31], as "insulin resistance inducing factors". Studies have shown that TNF-α leads to loss of IRS-1 and GLUT-4 expression [32] while IL-6 impairs insulin signaling by decreasing activation of IRS-1 [33]. Resistin too has been implicated in impairing glucose tolerance and insulin action [34].

These hypertrophic adipocytes also have an enhanced lipolytic activity and a diminished capacity to take up FFAs resulting in an abnormal and ectopic intracellular deposition of fats in liver, skeletal muscle and the pancreatic beta cells [8, 14]. This overload leads to an increased intracellular accumulation of FFAs and its metabolites such as long chain fatty acid coenzyme A (LCFA-CoA), diacylglycerol (DAG) and ceramides [35-37]. The end result is activation of the serine/threonine kinase, protein kinase C (PKC) [24, 35] which then phosphorylate insulin-

receptor substrates (IRS) on serine residues [37] rather than on tyrosine ones eliciting different responses among the different insulin target tissues. In the skeletal muscle these serine-phosphorylated forms cannot associate with and activate PI3K, resulting in decreased GLUT-4 activity [35]. In the hepatic cells, it results in reduced insulin stimulation of GS activity and decreased phosphorylation of the FOXO transcription factor thus activating transcription of the rate-controlling enzymes of gluconeogenesis [24]. The end result is increased hepatic glucose production and decreased skeletal muscle glucose uptake, which contribute immensely to increased plasma glucose levels.

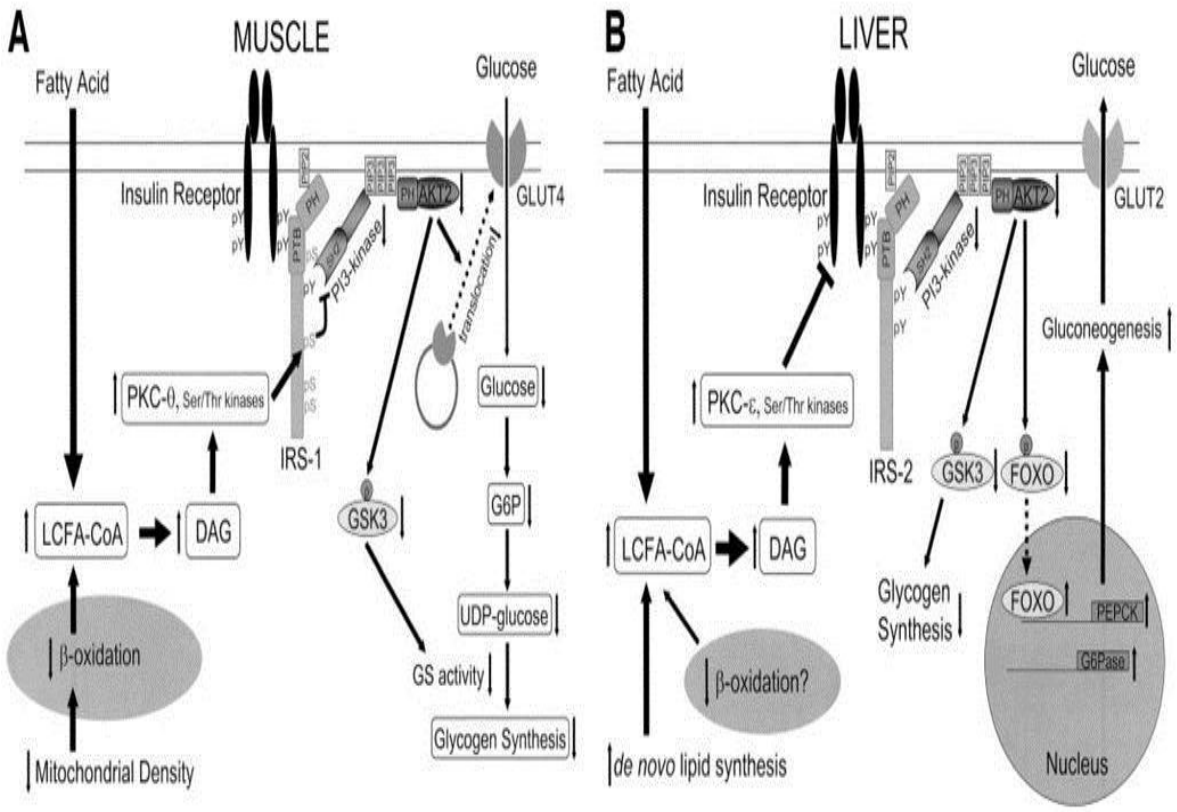

Figure 1.1. The molecular mechanism of insulin signaling in skeletal muscle and liver [24].

1.1.4 Dyslipidemia in insulin deficiency

DM is associated with a cluster of plasma lipid and lipoprotein abnormalities as a result of deficiency in insulin action and hyperglycemia. This is characterized by increased levels of very low density lipoproteins (VLDL), triglycerides (TG), reduced levels of HDL cholesterol [38] and a predominance of small-dense low density lipoprotein (LDL) [39]. This is especially the case in type 2 DM, a characteristic pattern referred to as diabetic dyslipidemia. However, elevated TG and decreased HDL levels have also been commonly observed in poorly controlled type 1 DM but usually resolves on insulin treatment [40]. Reduced insulin action results in an increased flux of FFA to the liver driving up the hepatic synthesis rates of VLDL and TG [41]. The secreted VLDL particles are metabolized to intermediate density lipoprotein (IDL) and further to LDL by lipoprotein lipase (LPL) which catalyses the rate-limiting step in lipoprotein metabolism [38, 41, 42]. Insulin deficiency diminishes the activity of LPL [43] and results in hypertriglyceridemia through a reduction in clearance of these lipoproteins. HDL is derived from metabolism of VLDL remnants and thus the reduced LPL activity causes a decrease in plasma HDL-cholesterol levels too [41, 43]. The rise in VLDL-TG promotes an abnormal exchange of TG and cholesteryl ester between VLDL, LDL and HDL, a process mediated by cholesterol ester transfer protein (CETP) [43]. As a result, ApoA-I easily dissociates from the TG-enriched HDL and is cleared rapidly from plasma thus reducing the availability of HDL [43]. These exchanges also result in increased small-dense LDL particles which readily undergo oxidative modifications promoting their uptake and retention in the vascular endothelium [44], a major risk factor for the development of cardiovascular complications. Regarded as a protective lipoprotein, HDL has been shown to have anti-inflammatory as well as antioxidant properties [38, 39, 43] although its

main function is in reverse cholesterol transport where cells unload cholesterol for eventual excretion in the bile via the liver [43].

1.1.5 Complications of diabetes mellitus

The escalating prevalence of DM is associated with an ever increasing burden of complications which are debilitating and even fatal. This situation is further compounded by delayed diagnosis which may increase the incidence and prevalence of diabetes thus exerting more pressure on the already stretched healthcare budgets and systems in the developing world. The complications of DM maybe acute or chronic.

Acute complications develop within a short duration and are extremely fatal in absence of effective treatment. These include diabetic ketoacidosis (DKA) and non-ketotic hyperosmolar state [5, 45] and are the most common hyperglycemic emergencies.

Chronic complications of DM result from changes in metabolism as a result of the hyperglycemic state. They are classified into macrovascular and microvascular complications. Progressively, they cause damage, dysfunction and ultimately failure of a number of organs. Macrovascular complications develop in the large arteries [42, 46] and manifest clinically as coronary artery disease (CAD) and stroke [46]. Microvascular complications occur as a result of damage to the microvasculature supplying the retinal, renal and neural tissues. This leads to diabetic retinopathy, nephropathy and neuropathy respectively. The diabetic foot syndrome, another complication arises from damage to both the micro and macro vasculature coupled with peripheral neuropathy [47].

1.2 Background: Diabetic Ketoacidosis (DKA)

Diabetic ketoacidosis (DKA) is a life threatening complication of diabetes mellitus. It is characterised by a triad of hyperglycemia, hyperketonemia and metabolic acidosis [48]. Hyperketonemia results from increased ketone body blood levels namely acetoacetate (AcAc), β-hydroxybutyrate (3HB) and acetone [48]. They serve as a major source of energy for tissues like the brain which are unable to utilize fatty acids [49, 50]. Ketone body levels are elevated in other conditions than DKA such as prolonged fasting and starvation [48, 49], prolonged exercise, consumption of a high fat diet and alcoholic intoxication [50]. It develops readily in pregnancy as a result of increased lipid energy metabolism [48] and also among neonates where they provide substrates for synthesis of cholesterol, fatty acids, and complex lipids like phospholipids and sphingolipids which are preferentially used over glucose as substrates for brain growth, myelination and lung phospholipids [51].

1.2.1 Epidemiology of DKA

Although commonly referred to as pathognomonic to type 1 DM, DKA might occur in type 2 DM but only under conditions of catabolic stress such as trauma, surgery or infections [8, 52]. Prevalence is high among the economically poor diabetic patients [53, 54] while reportedly, women are prone to DKA compared to men although poor compliance to insulin treatment has been attributed to this [54]. Pregnant women with diabetes face a higher risk of DKA as pregnancy is a state of relative insulin resistance and accelerated starvation as glucose is readily absorbed across the placenta with resultant increase in lipolysis and ketogenesis [55].

Mortality arising from DKA commonly occurs in children [54], teenagers and young adults with DM [52]. Among pregnant diabetic women, it is the leading cause of foetal loss as ketone bodies are readily transported across the placenta [55].

1.2.2 Precipitating factors of DKA

The most frequent precipitating factor of DKA among diabetic patients includes infections and omissions or inadequate insulin doses [52, 56, 57]. Infection remains the most common risk factor of DKA [52, 56] with urinary tract infections and pneumonia among the most frequently encountered [58]. A study by Mbugua *et al*. [59], identified infection and insulin omission as the most common precipitating factors. Other precipitating factors of DKA include myocardial infarction, cerebrovascular accidents, acute pancreatitis [56] alcohol abuse, pulmonary embolism, trauma [57] and use of medications such as steroids, thiazide diuretics and sympathomimetic agents [52] which affect carbohydrate metabolism.

1.2.3 Pathophysiology of DKA

Metabolic abnormalities in DKA occurs as a result of either absolute or relative insulin deficiency coupled with concomitant increases in the insulin plasma counter regulatory hormones, namely catecholamines, glucagon, cortisol and growth hormone. The catabolic nature of these hormones antagonises insulin's anabolic activity in the liver, adipose and skeletal muscle. Cortisol increases proteolysis which increases production of amino acids (alanine and glutamine) and also muscle glycogenolysis which increases lactic acid production, which serve as substrates for gluconeogenesis [60]. Catecholamines, glucagon and cortisol also stimulate the gluconeogenic enzymes like PEPCK [60]. As a result, glucose production is enhanced eventually leading to marked hyperglycemia, a key feature of DKA. Lack of insulin also leads to phosphorylation and activation of hormone sensitive lipase (HSL) in the adipose tissue [58] which promotes lipolysis and releases FFA, the main substrates for ketogenesis into the liver.

Hyperglycemia and increased ketone body production lead to osmotic diuresis with ketonuria and polyuria which coupled with hyperventilation cause dehydration. This leads to tissue

hypoxia and hyperlactidemia (Figure 1.2). Oxidative stress and free radicals which may exacerbate β-cell dysfunction also lead to production of pro-inflammatory cytokines which further provoke the release of insulin counter-regulatory hormones [45].

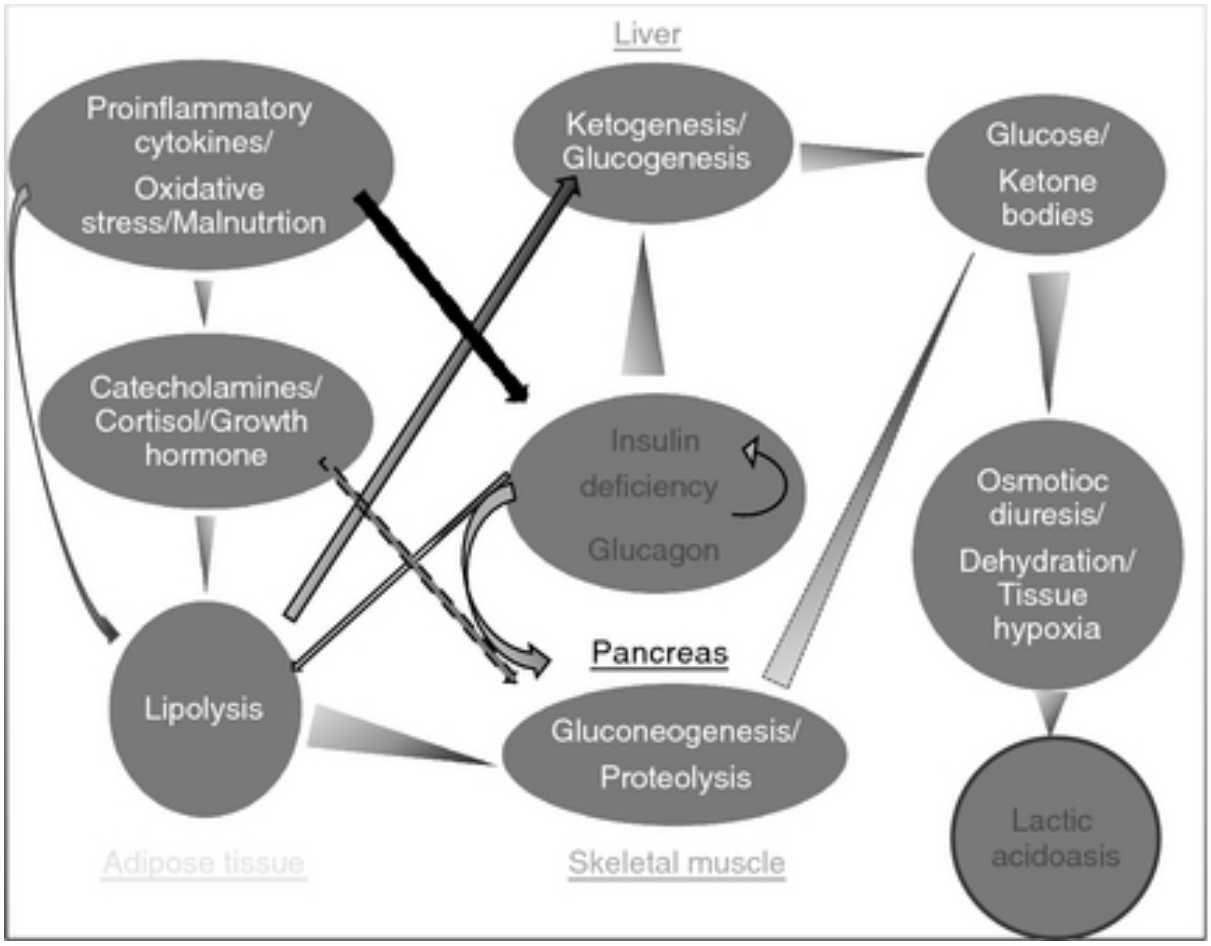

Figure 1.2. Schematic diagram of the pathophysiology of diabetic ketoacidosis (DKA) [3]**.**

1.2.3.1 Ketogenesis

Glucagon, plays a key role in ketogenesis by influencing both gluconeogenesis and ketone body formation. Increase in glucagon levels as a result of insulin deficiency triggers the phosphorylation of the enzyme acetyl co-enzyme (CoA) carboxylase which catalyses the conversion of acetyl CoA to malonyl CoA [48, 56, 58]. Malonyl CoA, the first committed

intermediate in the synthesis of long chain fatty acids inhibits fatty acid oxidation [57, 61] and facilitates FFA synthesis by inhibiting carnitine palmitoyl-O-transferase I (CPT I) [57, 58, 62]. CPT I is involved in transport of FFA across the mitochondrial membrane. Phosphorylation of acetyl CoA carboxylase reduces the production of malonyl CoA which leads to an elevation of CPT I levels and consequently, movement of FFA into mitochondria for oxidation and ketogenesis. Transport of FFA across the mitochondrial membrane occurs in the form of CoA after esterification to carnitine which is reversed by CPT II to form fatty acyl CoA which then enters the β-oxidative pathway (Figure 1.3). The acetyl CoA produced is used in the generation of AcAc and 3HB.

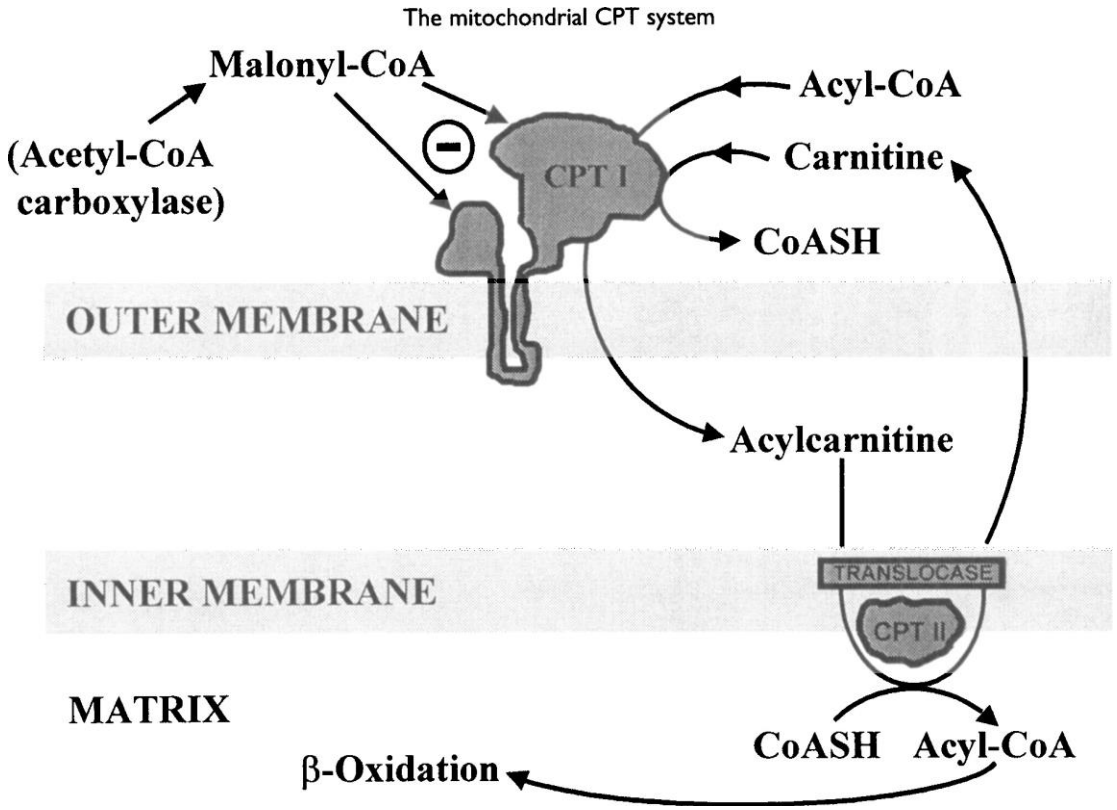

Figure 1.3. The mitochondrial carnitine palmitoyl-O-transferase (CPT) system showing the steps prior to beta oxidation [61].

After β-oxidation of fatty acids, acetyl CoA produced is the link to the TCA cycle. Normally, this channelling of acetyl CoA into the TCA cycle involves condensation with oxaloacetate [63]. However, under hypoglycemic conditions oxaloacetate is utilized preferentially in gluconeogenesis while the acetyl CoA is used in ketogenesis [63, 64] which occurs through a four step reaction pathway (Figure 1.4).

Figure 1.4. The sequential steps of ketogenesis (HMG-CoA pathway) [65].

The enzyme β-ketothiolase (acetoacetyl-CoA thiolase) catalyses the condensation of two acetyl molecules to form acetoacetyl CoA [63]. Acetoacetyl CoA is further condensed with another acetyl coA molecule forming hydroxyl methyl glutyryl CoA (HMG coA) in a reaction catalyzed by HMG CoA synthase [63, 65]. The HMG CoA formed is cleaved into AcAc and acetyl CoA by HMG CoA lyase [65]. AcAc is then reduced to 3HB by the enzyme 3HB dehydrogenase [63, 65], a phosphatidyl choline dependent enzyme with reduced nicotinamide adenine dinucleotide (NADH) being oxidized to NAD [48]. The enzyme HMG-CoA synthase is the rate limiting step in the synthesis of ketone bodies [65] and is therefore involved in the control of ketogenesis. The ratio of 3HB to AcAc generally reflects the mitochondrial redox state. Normally, the ratio is

relatively stable; however, acidosis in DKA leads to a reduced mitochondrial redox state which favors 3HB production [48, 50, 64, 66].

1.2.3.2 Ketolysis

This is the process by which ketone bodies are converted to energy that can be used in tissue metabolism. It involves the reconstitution of acetoacetyl CoA from acetoacetate by the rate limiting enzyme, succinyl CoA-oxoacid transferase (SCOT) and subsequently, cleavage of an acetyl group from acetoacetyl CoA by the enzyme methyl acetoacetyl thiolase (MAT) to form acetyl CoA [48]. The acetyl CoA is then channeled into the TCA cycle. Although SCOT has been detected in a number of tissues, it is lacking in the liver [67]. And this makes the liver unable to utilize the ketone bodies but instead derives its energy from the degradation of amino acids [63]. However, MAT activity is highest in the liver with substantial amounts also found in the kidney, heart, adrenal glands and skeletal muscle [67].

1.2.3.3 Acid-base balance in DKA

Metabolic acidosis in DKA occurs as a result of the increased production of AcAc and 3HB coupled with decreased utilization by peripheral tissues such as the skeletal muscle. Dehydration, a key feature of DKA results in reduced glomerular filtration rate (GFR) and hence an increased retention of the ketone bodies further worsening the acidosis. AcAc and 3HB being strong organic anions dissociate freely leading to an increased hydrogen ion load which rapidly exceeds normal bicarbonate ($HC0_3^-$) buffering capacity resulting in metabolic acidosis [52, 64]. The retention of these ketoanions eventually result in a high anion gap (AG) acidosis [68] as bicarbonate is replaced by AcAc and 3HB, anions which are not directly measurable.

1.2.4 Management of DKA

The true incidence of DM and by extension DKA in the developing world is largely unknown as the majority of patients remain undiagnosed. As a result, adequate management is still elusive. Therefore improvements in strategies on management or any of its related complication should aim to address this key issue.

1.2.4.1 Clinical presentation of DKA

Patient evaluation is critical in the management of DKA. It provides the necessary supportive information for preliminary diagnosis. The symptoms characteristic of poorly controlled diabetes may be observed for a number days; however, metabolic alterations typical of DKA usually evolve within a short period of time usually <24 hours. These include a history of polyuria, polydipsia and weight loss, abdominal pain, vomiting and an altered mental state [45, 56, 69]. Physical examination reveals Kussmaul (rapid and deep) breathing, a fruity acetone breath and features of dehydration [58, 69-71]. Particular attention should be paid to paediatric patients where presenting features of DKA may resemble other common illnesses such as bronchiolitis leading to misdiagnosis [54, 72]. This may delay treatment which worsens the prognosis as DKA progresses further to a more severe state.

1.2.4.2 Diagnosis of DKA

The initial laboratory evaluation of patients with suspected DKA includes determination of blood glucose, glycated haemoglobin, blood urea nitrogen/creatinine, serum and urine ketones, serum electrolytes with a calculated anion gap, serum osmolality, venous blood pH, complete blood count and electrocardiography. On suspicion of infection, blood and urinary bacterial cultures is performed.

Confirmatory laboratory diagnosis is based on a biochemical criteria characteristic of DKA. These include blood pH <7.3, serum bicarbonate concentration <15 mmol/L, blood glucose concentration >11 mmol/L with associated ketonemia and ketonuria [53, 73-75] and an increased anion gap (AG) [58, 76]. Based on these parameters, DKA has further been classified according to the severity (Table 1.2).

Categories	**Venous blood pH**	**Plasma bicarbonate (mM)**
Mild	7.2-7.3	15
Moderate	7.1-7.2	10
Severe	≤ 7.1	≤ 5

Table 1.2. Classification of DKA according to venous blood pH and plasma bicarbonate levels [3].

1.2.4.3 Treatment of patients with DKA

The treatment of DKA is multifaceted and should involve a well-structured approach. Treatment guidelines currently in use are developed in the Western world and might miss out on certain aspects unique to the Third world and thus caution should be observed. The main goals in the treatment of DKA are adequate correction of dehydration, acidosis and electrolyte imbalances while avoiding treatment related complications. Appropriate drug therapy is instituted in infection related causes.

1.2.4.3.1 Fluid replacement in DKA

Fluid replacement in DKA is aimed at correcting the fluid and electrolyte deficits. Over the years, a number of fluid management protocols have been developed. Despite these, it is still a major cause of concern as adequate assessment of fluid losses and optimal fluid therapy,

fundamental aspects in DKA treatment are still difficult to conclusively ascertain. Weight loss has been used as an indicator of dehydration [56, 72]. However, its main limitation is getting the accurate weight before the illness. Clinical presenting features too may be rendered unreliable. An example is hyperventilation in DKA due to metabolic acidosis which may lead to excessive drying of the oral mucosa resulting in over estimation of dehydration [77]. Biochemical variables too have been used to determine fluid losses. Hyperosmolality for example may preserve intravascular volume and thus maintain peripheral pulses, blood pressure and urine output until shock ensues [77] after severely underestimating dehydration. Currently, guidelines do not recommend clinical estimates of the volume deficits. Wolfsdorf *et al.,* [72], cited them as inaccurate and subjective and recommended using 5–7% dehydration in moderate DKA and 10% dehydration in severe DKA. However, to optimize therapy, it is imperative that all these variables of dehydration be correlated with rigorous monitoring.

Initial management involves rehydration to improve circulatory volume and tissue perfusion. This results in the restoration of extracellular fluid volume through administration of intravenous (IV) isotonic saline (0.9 % NaCl) [78, 79]. Normal saline is the fluid of choice [79, 80] and is also routinely used considering cost issues. Subsequent choice for fluid replacements is dependent on the state of dehydration, serum electrolyte levels and urinary output [56, 70]. Generally, if the corrected sodium levels are normal or elevated, hypotonic saline (0.45% NaCl) at a rate of 4-14 ml/kg/hr is administered [70, 81] which ensures a gradual correction of the fluid deficits as rapid correction is associated with a risk of cerebral oedema.

1.2.4.3.2 Insulin treatment in DKA

The discovery of insulin revolutionised the management of DKA. Prior to that, DKA was almost always a fatal occurrence. Insulin has the main role of reversing gluconeogenesis and

ketogenesis after which metabolism and renal excretion of the ketone bodies dissipates the acidosis. A key question always asked is; when is the right time to administer insulin? It is important that it be started after the patient has received initial fluid therapy because then, tissue perfusion will be restored and insulin distributed to all the target tissues. This should be about 1-2 hours after the initial fluid expansion [72]. A study by Edge *et al.*, [82], showed that early insulin administration, within the first hour was associated with an increased risk of cerebral edema. According to the study, the mechanism for this was related to the rapid changes in electrolytes which are greater during the initial phase of rapid rehydration when membrane electrolyte transport maybe most active.

Potassium levels must always be obtained before insulin therapy is started. This is because administration of insulin increases cellular potassium uptake by stimulating cell membrane Na^+/K^+ ATPase [83] leading to decreased plasma levels. In hypokalemia (potassium levels < 3.3 mmol/l) [56], potassium repletion should be initiated and insulin withheld as it worsens the hypokalemia which is life threatening as it may lead to cardiac arrhythmias [80, 84]. Insulin is also withheld in hypotensive patients with severe hypoglycaemia as it may lead to vascular collapse from the rapid decrease of blood glucose secondary to insulin administration which causes movement of fluid from extracellular to intracellular space [80]. Among these patients, stabilization of the blood pressure should be the main priority.

Studies and guidelines on the management of DKA recommend the administration of low dose insulin via intravenous (IV) route as the most preferred route of delivery [53, 75, 85]. Compared to subcutaneous (SC) and intramuscular (IM) routes, IV administration has been shown to possess a shorter half-life thus easy to achieve accurate titration and a linear predictable fall in blood glucose [86]. Furthermore, erratic absorption is observed in dehydrated patients with IM

and SC routes compared to IV administration [87]. Over the years, studies have also shown that low dose insulin is effective in treatment of DKA by achieving a steady fall of plasma glucose levels while reducing risk of hypoglycaemia and hypokalemia [56, 88]. Another practise that still elicits debate is the use of a priming dose during insulin IV infusion. Some studies advocate for it [86] but concerns still exist over its clinical effectiveness. A number of recent studies have indicated that it confers no added advantage as long as continuous IV infusion is adequate [89, 90] and may further potentiate the risk of undesirable effects such as hypokalemia and cerebral edema [72, 90]. The low dose continuous IV insulin infusion is administered at 0.1 U/kg/h) [56, 80, 87]. Correction of hyperglycemia generally occurs faster than acidemia. Therefore, once plasma glucose reaches about 14 mmol/l, the insulin infusion rate is halved as 5% dextrose is added to the infusion fluid [56]. This avoids the complication of hypoglycemia until complete resolution of ketoacidosis. Thereafter, transition to SC insulin at 0.1 U/kg every 4 hours should be initiated at least 1-2 hours before IV infusion is stopped [91] because of the slow onset of action via SC route. The established DM patients can then resume their previous insulin doses with necessary adjustments while new dose regimens are worked out for the newly diagnosed.

Despite continuous IV insulin administration being the most preferred route, SC and IM may still play a role in uncomplicated DKA and in resource poor settings where access to equipment such as infusion pumps is a challenge. Although tedious, administration of rapid-acting insulin analogs via SC and IM routes every 1-2 hours has been shown to be almost as effective as IV administration in resolution of hyperglycemia and ketoacidosis [72]. This then allows for treatment in the emergency departments, general wards and clinics especially in resource limited settings such as the developing world. This significantly reduces costs by eliminating need for infusion pumps and hospitalization costs.

1.2.4.3.3 Serum electrolyte levels in DKA

Determination of serum potassium levels is a key laboratory test in DKA and must be performed on initial presentation, before commencement of treatment. Serum potassium levels may be normal, elevated or decreased, although total body deficits of about 3–6 mmol/kg are usually recorded among adults [53, 80]. Hyperglycemia causes extracellular hyperosmolality which causes water and potassium shifts into the extracellular space resulting in the normal or elevated levels which is further enhanced by insulin deficiency [92]. Insulin normally promotes shift of potassium into the intracellular space. However, deficits are still recorded. This is as a result of the hyperglycemia induced osmotic diuresis which enhances urinary losses [92].

Current treatment guidelines recommend that to prevent hypokalemia, replacement therapy should be initiated once levels fall below 5.5 mmol/l [93]. Above this, replacement should be withheld [80] as this may cause hyperkalemia which may cause cardiac arrest. During the replacement, it is recommended that potassium levels and the electrogram [ECG] be determined frequently [92, 94]. Adequate urine output should always be ensured [94] as this avoids hyperkalemia as a result of decreased renal excretion. It is therefore evident that initiation of potassium replacements with undetermined levels and compromised renal function may result in fatal outcomes. According to the clinical guidelines by De Beer *et al.* [93], administration of 40 mmol/l of potassium is adequate when the serum levels are less than 3.3 mmol/l but when between 3.3 and 5.5 mmol/l, 20 to 40 mmol/l is appropriate. Potassium is given as an infusion mixture of phosphate and chloride [71] which avoids the risk of excess chloride administration.

Phosphate levels are normal or elevated on initial presentation of DKA but are reduced by insulin treatment [93] which promotes entry into the cells. Osmotic diuresis further enhances the losses

[92] through increased urinary losses. However, concern about phosphate supplementation exists as the benefits are still unclear. Furthermore, excessive administration is associated with reported risk of hypocalcemia [58] with tetany, soft tissue calcification [92] hence caution is advised. Patients with serum phosphate levels <1 mg/dl experience tissue hypoxia [94] due to a reduction of 2, 3-diphosphoglycerate (2, 3-DPG) in the red blood cells which increases affinity of haemoglobin to oxygen thus decreasing oxygen supply to tissues [52]. It is therefore recommended that such patients be nebulized. To correct these low levels, 20-30 mmol/l potassium phosphate is added to the infusion fluids and given over several hours [69]. However, excess phosphate administration should be avoided.

1.2.4.3.4 Serum bicarbonate replacement in DKA

Bicarbonate therapy in DKA is still controversial. Recent studies have not demonstrated any improvement on metabolic recovery on its administration [93, 95, 96]. Furthermore, its use is associated with serious life threatening adverse effects such as hypokalemia, paradoxical worsening of central nervous system acidosis [56, 69] and an increased risk of cerebral edema [89, 94]. Mostly, adequate hydration and insulin therapy automatically corrects the acidosis [91] hence no need for alkalinisation. Insulin inhibits ketogenesis and promotes ketoanion metabolism consuming protons along the way while regenerating bicarbonate and spontaneously correcting the acidosis [53, 92].

However, bicarbonate may be recommended in patients with severe metabolic acidosis (pH <7.0) [69] to whom 40 mmol/l is administered until the pH rises to 7.0 [94] although studies on this especially at the low pH are still lacking. Therefore, caution should be exercised as the rise in pH may cause reversal of the protective Bohr Effect which releases oxygen during acidosis by

decreasing haemoglobin's affinity [52]. This may cause tissue hypoxia and worsen the patient's condition.

1.2.5 Complications of DKA

Cerebral edema is a serious and fatal complication of DKA. It is a rare condition [77] mostly prevalent among children and adolescents [97]. Reported incidences in both newly diagnosed and established cases are 0.7% in the United Kingdom [98], 2% in Australia [99] and 0.9% in Northern America [100]. However, in Africa; the true incidence is unknown as majority of the patients die undiagnosed. The pathogenesis is not clearly understood but accumulation of water in the brain tissue [101], in addition to breakdown of blood brain barrier, metabolic abnormalities or hyponatremia which are common in children under the age of 5 years especially those with severe acidosis and hypocapnia [73].

Clinically, the presenting features include decreased level of consciousness and headache followed by seizures, sphincter incontinence, pupillary changes, papilledema, bradycardia and respiratory arrest [69]. Mortality is very high (>70%) [70] while chances of recovery without permanent disabilities are low [70, 73]. Therefore, measures to reduce the risk should be prioritized. These involve gradual correction of blood glucose levels, fluid and sodium deficits.

Murcomycosis, an opportunistic fungal infection is a rare but fatal complication of chronic ketoacidosis, involving the respiratory tract and sinuses [102]. When disseminated it may further complicate cerebral edema in these patients [73].

Other serious complications of DKA include renal failure which although rare may necessitate dialysis, increased thromboembolic events known to occur in children following dehydration, central venous catheter placement in ICU or idiopathic elevation of von Willebrand factor [73]. Acute pancreatitis is also common in children with severe DKA and unresolving pain during

treatment [73, 103]. Rhabdomyolysis commonly associated with renal failure may be experienced by paediatric patients with severe hyperglycemia, high osmolality and hypophosphatemia [73].

1.2.6. Limitations of the current treatment guidelines of DKA

Major advances have been made in the management of DKA. However, despite these, high morbidity and mortality rates from DKA are still recorded. This is as a result of the numerous challenges experienced especially in the developing world. These include erratic supply of insulin, lack of proper storage facilities such as refrigerators. This is coupled with shortage of supplies such as syringes, infusion pumps and blood glucose measuring equipment due to poor healthcare systems. As a result, the economic cost of DM management has become unaffordable to the people as they are forced to acquire them individually.

This is in addition to the side effects of insulin such as allergic reactions like erythema, pruritus, indurations at the application site and lipoatrophy [104] which further compromise compliance, worsen glycemic control and complicate the disease process. As a result, the search for novel therapies which are safer, cheaper and therapeutically effective has been hastened shifting the focus of research to plants and plant products which have been used throughout human history to cure and prevent diabetes.

1.3 Medicinal use of plants in diabetes

Plants have been used as medicine since ancient times. It is believed that as early as the Neanderthal man, they had special healing powers [105, 106] with the earliest records of plant use as medicine found among Chinese [106], Egyptians and also in the code of Hammurabi [107]. They are particularly important in the developing world where traditional medical knowledge systems have sustained generations. This is further emphasized with reports

indicating that 80% of the world population is dependent on traditional medicine, 90% of which is derived from plants [108].

A lot of progress has been made concerning knowledge of bioactive components in plant foods and the role they play in health. Their consumption is no longer purely for their nutritive value but also as nutraceuticals which play an important role in the maintenance of health. These nutraceuticals include the polyphenols, phytoestrogens, phytosterols, phytates and polyunsaturated fatty acids [109]. The polyphenols, mainly found in fruits and vegetables are among the most important sources of bioactive components of the human diet [109]. Flavonoids, ubiquitously found in the plant kingdom are polyphenols and have been the subject of immense scientific interest. They are widely consumed for their biological and pharmacological properties such as antioxidant, anti-inflammatory, anti-allergic, antiviral, antibacterial, anti-mutagenic and anti-carcinogenic activities [110].

Studies have been done to demonstrate the antidiabetic effects of plants and thus justifying their use in traditional medicine. Among these plants include *Vernonia amygdalina*, *Hypoxis hemerocallidea*, *Sclerocarya birrea*, *Psidium guajava*, *Sutherlandia frutescens* [111] and *Citrus paradisi* [112] commonly known as the grapefruit. The grapefruit was first introduced as a weight loss food as part of the Hollywood diet of the 1930s, which recommended half of a fresh grapefruit before every meal for 12 days [113]. Recently, Fujioka *et.al*. [114]**,** showed that grapefruit consumption leads to weight loss which is even more significant when it is consumed just before meals. This is believed to be due to its combined effects of low dietary energy density and high fibre content [115]. Based on these effects, the grapefruit and its constituents may therefore be beneficial in patients with DM and its related complications like DKA.

1.3.1 The grapefruit

Flavonoids constitute the most abundant bioactive constituent of the grapefruit, a popular worldwide fruit [116]. Commercially, the grapefruit is also of great importance in the juice industry and also as a source of essential oils and pectin [117]. However, claims of its medicinal properties have led to increased consumption and interest from researchers. Among the many reported medical uses include anti-diabetic [116], anti-atherosclerotic, anti-carcinogenetic [118] anti-inflammatory, anti-proliferative, and antimicrobial properties [119].

1.3.1.1 Phytochemistry of the grapefruit

The grapefruit is rich in a large number of phytochemicals. These include the flavonoids, furanocoumarins (FC) [120, 121], limonoid aglycones, glucosides, ascorbic acid, folic acid, glucaric acid, carotenoids, pectin and potassium [116]. Flavonoids constitute the most abundant bioactive constituent of the grapefruit [116] and have a generic structure made up of 2 aromatic rings, A and B linked by a heterocyclic ring C as shown in figure 1.5. Based on changes on ring C, they are generally classified into six; flavonols, flavones, flavanones, anthocyanins, isoflavones and flavans as shown in figure 1.6. Flavanones are the most abundant making up 98% of the total flavonoid content [122]. In grapefruit, flavonoids usually exist as glycosides where a sugar molecule is attached to the aglycone. Two types of glycosides are already classified; neohesperidosides and rutinosides [123]. Neohesperidosides (naringin, neohesperidin, poncirin and neoeriocitrin) consist of a flavanone with neohesperidose (rhamnosyl-α-1,2 glucose) and have a bitter taste, while rutinosides (hesperidin, narirutin and didymin) have a flavanone and a rutinose (rhamnosyl-α-1,6 glucose) and are tasteless [123]. On ingestion, the glycosides are hydrolysed into their aglycones and sugars by the action of intestinal flora [116,

120, 124]. Naringin, the glycoside of naringenin is the most abundant flavonoid in GFJ and is majorly responsible for its distinctive aroma and pungent taste [124].

The major furanocoumarins (FC) present in the grapefruit include bergamottin, 6',7'-dihydroxybergamottin (DHB) [125, 126]. These have been extensively studied as the causative agents of grapefruit-drug interactions. Grapefruit juice (GFJ) also contains FC dimers [125, 127] also known as spiroesters or paradisins which are present at lower concentrations than FC but have shown potent in vitro enzyme inhibitory activity [121]. Variations in the concentration of these flavonoids and FC in grapefruit have been reported. A number of factors have been cited as the cause among them the grapefruit variety, method of processing, storage conditions [121] and time of harvest [116].

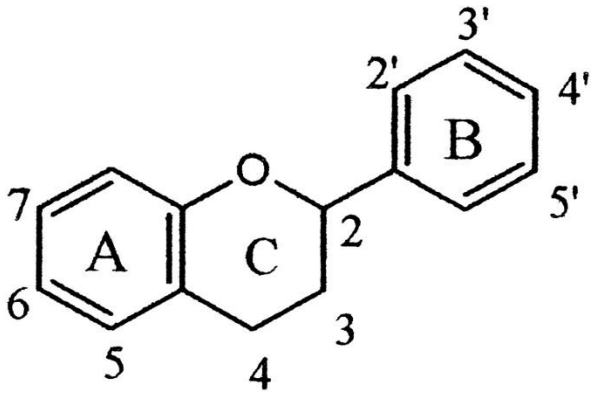

Figure 1.5. The generic structure of flavonoids [128]

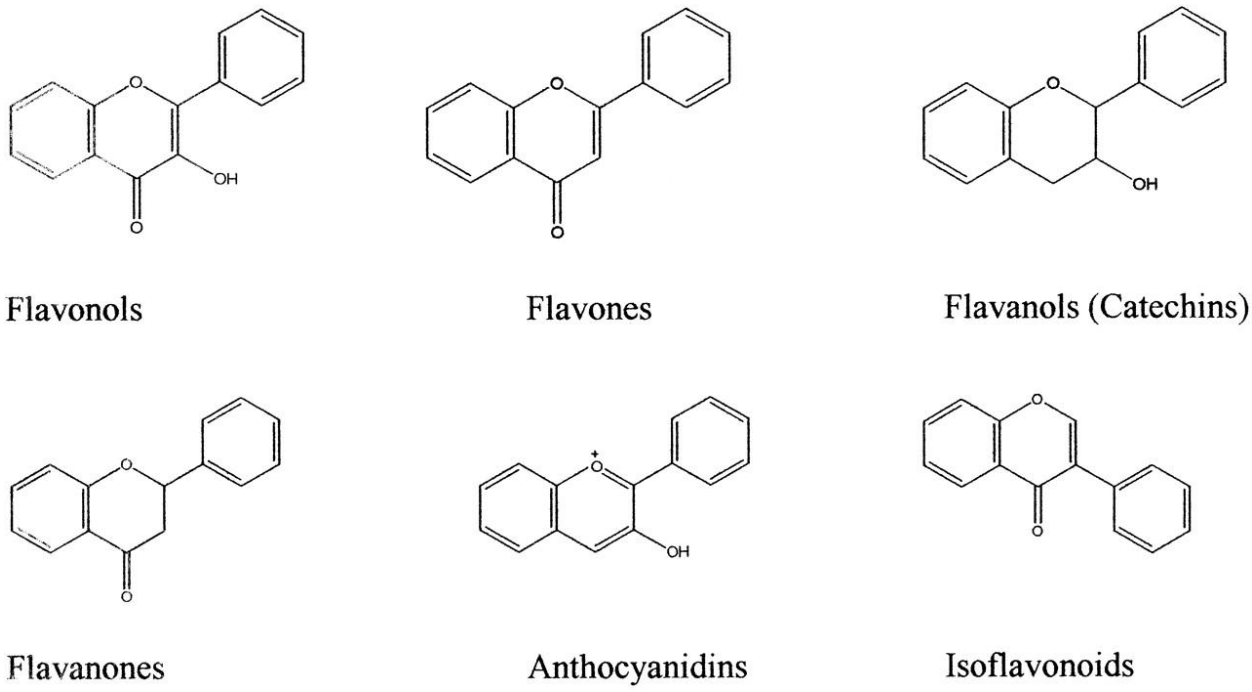

Figure 1.6. The six main classes of dietary flavonoids in citrus fruits [128].

1.3.1.2 Naringin and diabetes

As the burden of obesity increases globally to epidemic proportions, strategies are being employed to try and curb it. In DM, the benefits of weight reduction are evident as its previously been shown that moderate weight loss among patients with type 2 DM was associated with improvements in glycemic control [129]. Further reports indicate that weight loss is also associated with improvements in insulin sensitivity, dyslipidemia and blood pressure [130]. The grapefruit is particularly associated with reduction of central/truncal obesity [113], a key characteristic of the metabolic syndrome and a major predisposing factor of type 2 DM. This has been attributed to its flavonoids such as naringin which not only improve abdominal obesity but other risk factors such as insulin resistance [131, 132], the main biochemical disturbance in metabolic syndrome.

Dietary flavonoids have been shown to possess anti-diabetic and anti-dyslipidemic activities with researchers currently trying to identify the exact roles played by these flavonoids. Naringin has been shown to possess anti-diabetic and anti-dyslipidemic activities [133].

These observations suggest that naringin improves glycemic control and might play a major role in treatment of DKA. As a result, this study proposed to investigate the effects of naringin consumption on DKA in type 1 diabetes rat model.

1.4 AIM: To investigate the putative roles of naringin in the pathogenesis of diabetic ketoacidosis (DKA) in a diabetic rat model.

1.4.1 Objectives

1. To establish a type 1 diabetes rat model with diabetes ketoacidosis.
2. To investigate the effects of naringin on diabetic ketoacidosis.
3. To elucidate the underlying mechanisms involved in the activity of naringin in the diabetic ketoacidosis.

Chapter Two

2.0 Material and methods

2.1 Chemical and reagents

The chemicals and reagents used in the study were purchased from Sigma-Aldrich Pty. Ltd., Johannesburg, South Africa. Reagents and drugs used included naringin, D-glucose, streptozotocin, citrate and phosphate buffers, potassium chloride, sodium chloride, sulphuric acid, hydrochloric acid.

Insulin (Novo Nordisk®, Norway), normal saline, portable glucometers and glucose test strips (Ascencia Elite™, Bayer Leverkusen, Germany) were purchased from a local pharmacy. Halothane and other accessories were provided by the Biomedical Research Unit (BRU) of the University of KwaZulu-Natal, Durban, South Africa.

2.2 Study design

2.2.1 Ethics approval

The experimental protocol for the study was approved by the Animal Ethics Committee of the University of KwaZulu-Natal, reference number 106/13/animal.

2.2.2 Animal treatment

Male Spague-Dawley rats of 200-300g body weight were divided into 5 groups and housed seven rats per cage. Animals were given free access to standard commercial chow and drinking tap water *ad libitum*. The rats were maintained on a 12 hour dark-to-light cycle of 08.00 to 20.00 hours light in an air controlled room (temperature 25 ±2ºC, humidity 55% ±5%) and were handled humanely, according to the guidelines of the Animal Ethics Committee of University of KwaZulu-Natal

2.2.3 Induction of diabetes

Diabetes was induced by intraperitoneal (IP) injection of 60 mg/kg body weight of streptozotocin (STZ) for type 1 diabetes. This was prepared by dissolving the STZ in 0.2 ml citrate buffer, pH 4.5 and was administered after an overnight starvation of the rats. Three days after STZ administration, development of diabetes was confirmed by tail pricking to analyse the blood glucose levels. Rats with random blood glucose levels above 11 mmol/L were considered diabetic and were included in the study [134].

2.2.4 Experimental design

Type 1 DM was induced in group 3, 4 and 5. Groups 2 and 4 were orally treated with 50 mg/kg of naringin. Group 3 was treated with regular insulin (4 IU/kg) subcutaneously twice daily. Groups 1 and 5 were treated with 1 ml distilled water via gastric gavage. Insulin treatment was only initiated after the confirmation of the presence of diabetes. Animal weights and water consumption were measured daily. On day 40, each animal was placed in metabolic cages and urine was collected over a 24 hour period, measured and recorded. On treatment day 42, halothane overdose was used to sacrifice the rats. Blood samples were collected via cardiac

puncture for plasma insulin, electrolytes and ketone body analysis while liver samples were excised, snap-frozen in liquid nitrogen and stored at -80˚C for further analysis.

Group	Diabetic status	Treatment
1	Negative	Distilled water
2	Negative	50 mg/kg naringin
3	Positive	Regular insulin
4	Positive	50mg/kg naringin
5	Positive	Distilled water

Table 2.1. Animal treatment schedule

2.3 Methods

2.3.1 Blood glucose testing

Fasting blood glucose tests were done on treatment days 0, 14, 28 and 41 respectively whereas glucose tolerance test (GTT) was done on day 41. Blood glucose concentrations were determined after tail-pricking and were analysed by a portable glucometer. Prior to GTT, all animals were starved overnight. Fasting blood glucose (FBG) for GTT was then determined after intra peritoneal (IP) administration of 3.0 mg/kg body weight of glucose in normal saline. The blood glucose concentrations were measured at times 0, 15, 30, 60, 90 and 120 minutes in all treatment

groups. Area under the curve (AUC) was calculated from blood glucose time curves and presented as AUC units (mmol/L×minutes).

2.3.2 Determination of plasma insulin

An ultra-sensitive rat insulin enzyme-linked immunoassay kit (DRG Diagnostics, Marburg, Germany) was used to analyse the plasma insulin levels as per the manufacturer's instructions. Briefly, 25 µl of samples/standards, 100 µl of enzyme conjugate were added to a 96-well plate and incubated on a plate shaker at 900 rpm for 2 hours at 25˚C. The plate was washed 6 times each with 700 µl of wash buffer after which 200 µl of substrate TMB was added into each well and incubated for 15 minutes at 25˚C. Stop solution (50 µl) was added to each well and incubated for 5 minutes on a plate shaker before measuring the optical density in a microplate reader (EZ Read 400, biochrom®) at 450 nm.

2.3.3 Hepatic glycogen assay

Hepatic glycogen content was measured by the modified method of Seifter *et al.,* [135]. Briefly, the liver tissue was homogenised in 1.0 ml of 30% potassium hydroxide saturated with sodium sulphate. The homogenate obtained was dissolved by boiling in a water bath (100˚C) for 30 minutes, vortexed and cooled in ice. Glycogen was then precipitated with 2.0 ml of 95% ethanol, vortexed, incubated in ice for 30 min and later centrifuged at 550 g for 30 min. The glycogen pellets obtained were then re-dissolved in 1 ml of distilled water which was thereafter treated with 1 ml of 5% phenol and 5 ml of 96-98% sulphuric acid respectively. This was incubated in ice bath for 30 min and the absorbance measured at 490 nm using a spectrophotometer (Genesys 20, Thermo Spectronic®). Glycogen content was expressed as mg/g liver protein.

2.3.4 Determination of serum electrolyte and pH level

Serum sodium (Na^+), potassium (K^+), chloride (Cl^-), bicarbonate ($HC0_3^-$) and blood pH levels were determined using an automated chemistry analyser (Beckman Coulter, Synchron LX20 Clinical Systems, California, USA) while the pH was determined using a pH/ blood gas analyser (Chiron Diagnostics, Halstead, Essex, UK).

The anion gap (AG) was calculated using the formula:

$$AG = \{[Na^+] + [K^+]\} - \{[Cl^-] + [HC0^{3-}]\ [68]$$

2.3.5 Analysis of serum ketone body levels

Serum and urine ketone body levels were determined using the spectrophotometric enzymatic assay kit (Enzychrom ^{TM}, BioAssay systems, EKBD-100) according to the manufacturer's instructions. Briefly, 8 mM of acetoacetate (AcAc) standard was prepared by mixing 5 µl AcAc standard provided with 45 µl distilled water. 5 µl of the 8 mM AcAc standard and 5 µl distilled water were then transferred in separate wells of a clear flat bottomed 96 well plate.5 µl of each sample was also transferred into 2 wells, one sample well and one blank well. A working reagent for water, standard and sample wells was prepared by mixing 195 µl AcAc buffer, 8 µl AcAc reagent and 0.5 µl hydroxybutyrate dehydrogenase (HBDH) enzyme for each well. A blank reagent was also prepared by mixing for each blank well, 195 µl of AcAc buffer and 8 µl AcAc reagent (no enzyme). 195 µl of the working reagent was added to the water, standard and sample wells while 195 µl blank reagent was added to the sample blank wells with gentle tapping to ensure mixing. The solutions were then incubated at room temperature for 5 minutes and optical density (OD) read at 340 nm using a spectrophotometer (Genesys 20, Thermospectronic). The concentration was determined according to the equation below:

$$[AcAc] = \frac{OD\ blank - OD\ sample}{OD\ water - OD\ standard}$$

A similar procedure was repeated for the 3-β hydroxybutyrate (3HB) assay using the 3HB buffer, 3HB reagent and 3HB standard. The concentration was calculated as follows:

$$[3HB] = \frac{OD\ sample - OD\ blank}{OD\ standard - OD\ water}$$

2.4 Statistical significance

The data was presented as mean ± SD and analyzed by GraphPad Prism Software Version 5.0. Mann–Whitney tests and/or Student t-tests were applied to the results to determine statistical significance. Values of $P < 0.05$ were taken to imply statistical significance.

Chapter Three

3.0 Results

3.1 Animal growth change during treatment period

The diabetic rat groups exhibited significant ($p<0.0001$) weight loss compared to the normal control group. In addition, diabetic rats treated with insulin and naringin exhibited significantly ($p<0.0001$) improved weight gain compared to the non-treated diabetic rat group. Treatment of the normal non diabetic rats with naringin had no significant change in weight gain in comparison to the normal control (Figure 3.1).

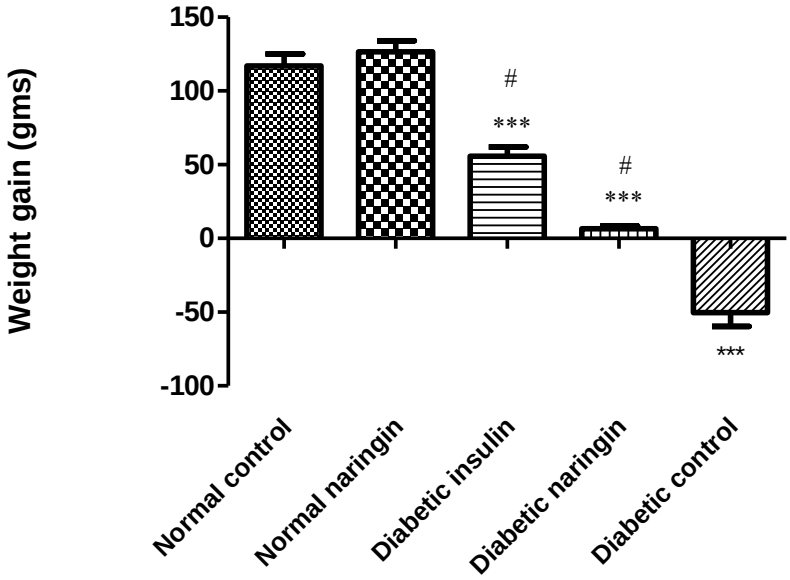

Figure 3.1. Animal weight changes during treatment period (***$p<0.0001$ compared to normal control, #$p<0.0001$ compared to diabetic control).

3.2 Water consumption during treatment period

The average daily water consumption was consistently and significantly ($p < 0.0001$) higher in all diabetic rats compared to normal control group. However, water consumption was significantly ($p<0.0001$) reduced in the insulin and naringin treated groups compared to the diabetic control group (Figure 3.2).

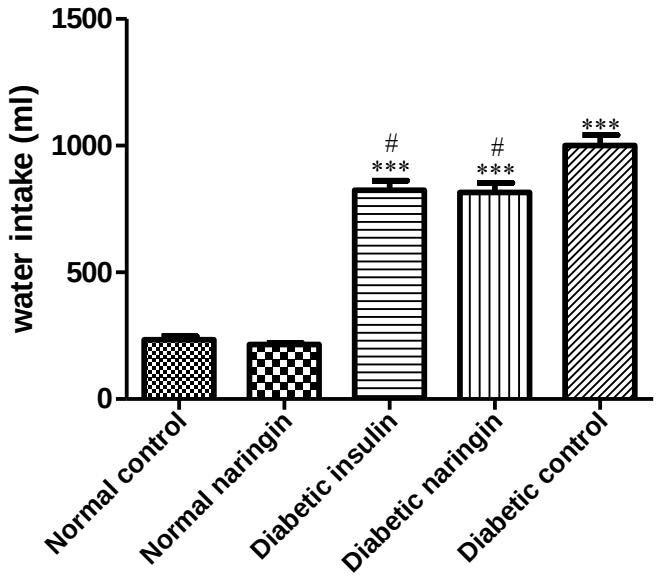

Figure 3.2. Water consumption during treatment period (***$p<0.0001$ compared to normal control, #$p<0.0001$ compared to diabetic control).

3.3 Urine output

The urine output was significantly ($p<0.0001$) elevated among all the diabetic groups in comparison to the normal control rat group. Treatment of the diabetic group with insulin significantly ($p<0.05$) reduced the urine output as compared to the diabetic control group. However, naringin treatment did not have any significant effect on the diabetic animals (Figure 3.3).

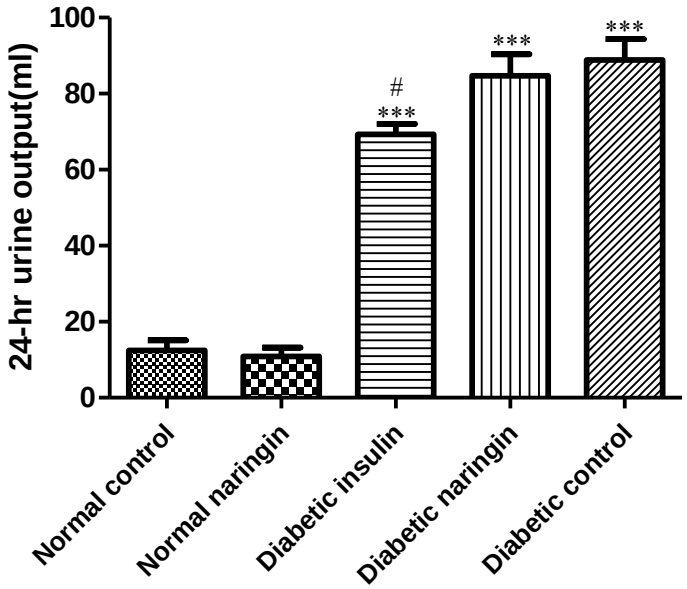

Figure 3.3. 24-hour urine output (***$p< 0.0001$ compared to normal control, # $p< 0.05$ compared to diabetic control).

3.4 Blood glucose level measurements

FBG levels were significantly higher ($p<0.0001$) in all diabetic rat groups compared to the normal non-diabetic control. However, compared to the diabetic control group no treatment corrected the FBG levels (Figure 3.4.A).

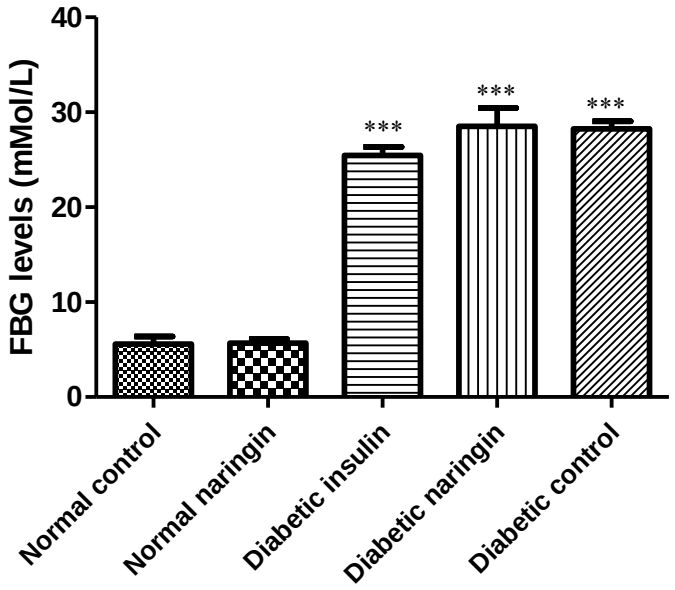

Figure 3.4.A. Fasting blood glucose (FBG) levels (***$p<0.001$ compared to the normal control group).

Calculated AUCs showed that the diabetic groups ($p<0.0001$) significantly increased AUCs compared to normal control group confirming impaired glucose tolerance. However, compared to the diabetic control group, naringin and insulin treatment did not improve the AUCs among the diabetic groups (Figure 3.4.B).

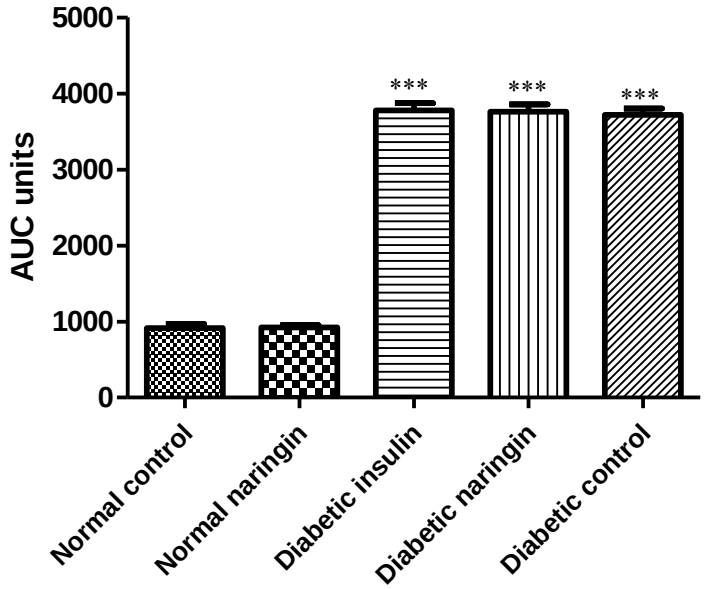

Figure 3.4.B. Calculated AUC for OGTT (***$p<0.0001$ compared to the normal control group).

3.5 Fasting plasma insulin concentration

Fasting plasma insulin concentration (FPI) were significantly ($p<0.0001$, $p<0.01$ and $p<0.01$) lower in the diabetic control, diabetic insulin and diabetic naringin compared with the normal control group. Treatment of the diabetic rats with insulin and naringin improved the plasma FPI levels although no significance was recorded compared to the diabetic control. Naringin treatment significantly ($p=0.0003$) elevated the plasma insulin levels among the non-diabetic rats in comparison to normal control (Figure 3.5).

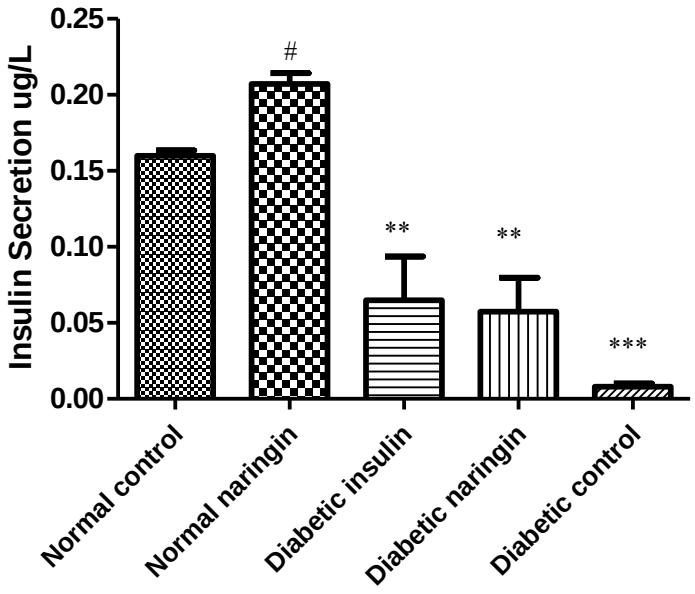

Figure 3.5. Plasma insulin levels (***$p<0.0001$, **$p<0.01$ #$p=0.0003$ compared to normal control).

3.6 Hepatic glycogen levels

The diabetic control rat group had a significantly ($p<0.0001$) reduced hepatic glycogen content compared to the non-treated diabetic control group. Treatment of the diabetic rats with insulin and naringin showed significant ($p<0.01$ and $p<0.05$ respectively) increases in hepatic glycogen content as compared to the diabetic control group. Naringin treatment of normal rats showed significant ($p<0.05$) elevation of glycogen content compared to the normal control (Figure 3.6).

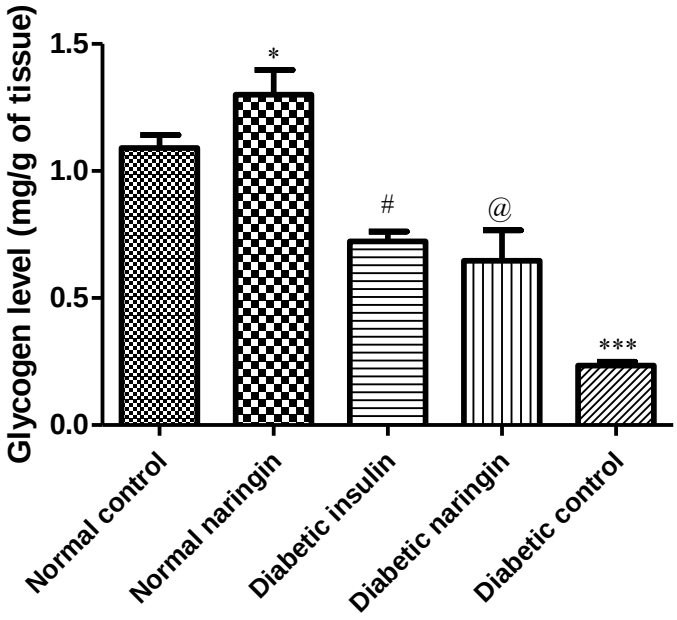

Figure 3.6. Glycogen levels in hepatic tissue (***$p< 0.0001$, *$p< 0.05$ compared to normal control, #$p< 0.01$, @ $p<0.05$ compared to diabetic control).

3.7 Serum ketone body levels

The 3HB levels were significantly ($p<0.001$) elevated in the diabetic control rat group compared to the normal control group. Insulin and naringin treatment significantly ($p<0.001$ and $p<0.05$ respectively) reduced serum 3HB concentration in the diabetic groups compared to the diabetic control group (Figure 3.7.A).

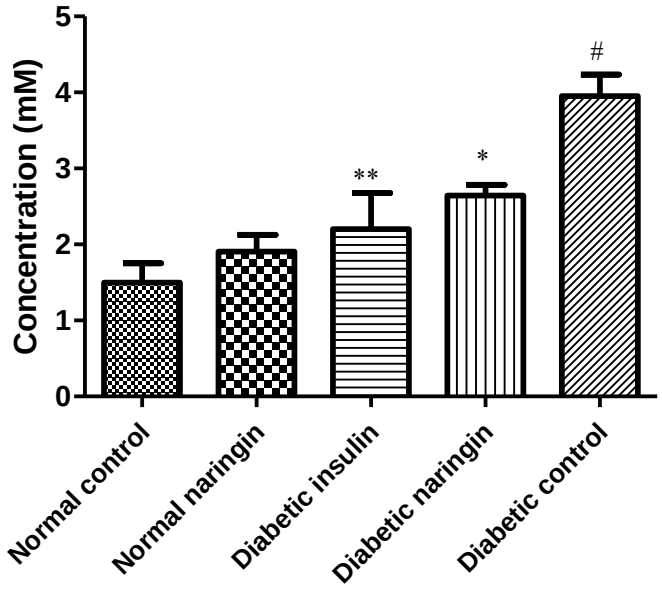

Figure 3.7.A. Serum 3-β-hydroxybutyrate (3HB) levels (#$p< 0.001$ compared to normal control, **$p<0.01$ and *$p<0.05$ compared to diabetic control).

Serum AcAc levels were significantly ($p<0.0001$) elevated in the diabetic rats compared to normal control rats. Naringin treatment significantly ($p<0.05$) reduced the blood AcAc levels in diabetic rats compared to non-treated diabetic control (Figure 3.7.B).

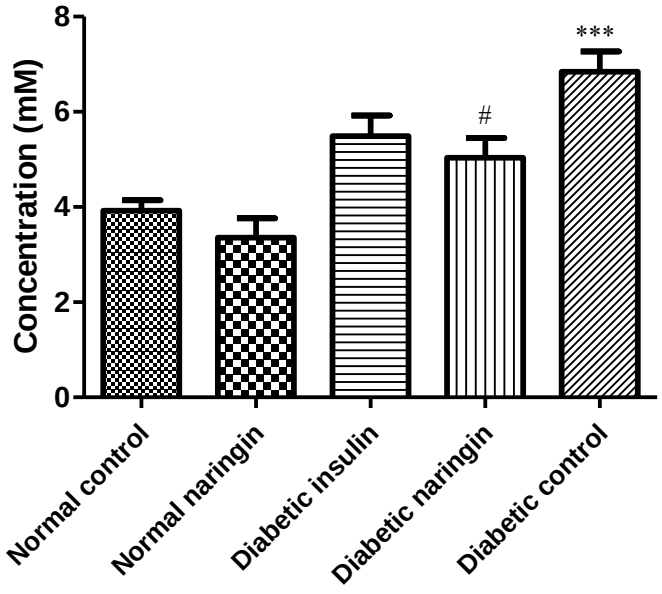

Figure 3.7.B. Serum acetoacetate (AcAc) levels (***$p<0.0001$ compared to normal untreated control, #$p<0.05$ compared to diabetic control).

The total ketone body (TKB) levels was also significantly ($p<0.0001$) elevated in the diabetic control rats compared to the normal control rats. Treatment with insulin and naringin significantly ($p<0.001$ and $p<0.001$ respectively) decreased the TKB levels in diabetic groups compared to the diabetic control group (Figure 3.7.C).

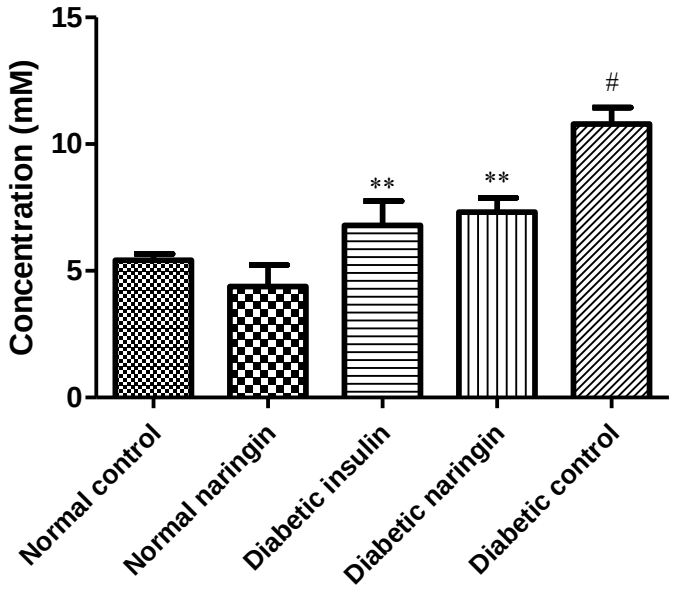

Figure 3.7.C. Total ketone body levels in serum (**$p<0.01$ compared to the diabetic control group, #$p<0.0001$ compared to normal control group).

The 3HB:AcAc ratio was significantly ($p<0.05$) elevated in the diabetic rat groups as compared to the normal rat control group. However, none of the treatment administered to the diabetic rat groups produced any significant reduction of the ratio compared to the diabetic rat control group (3.7.D).

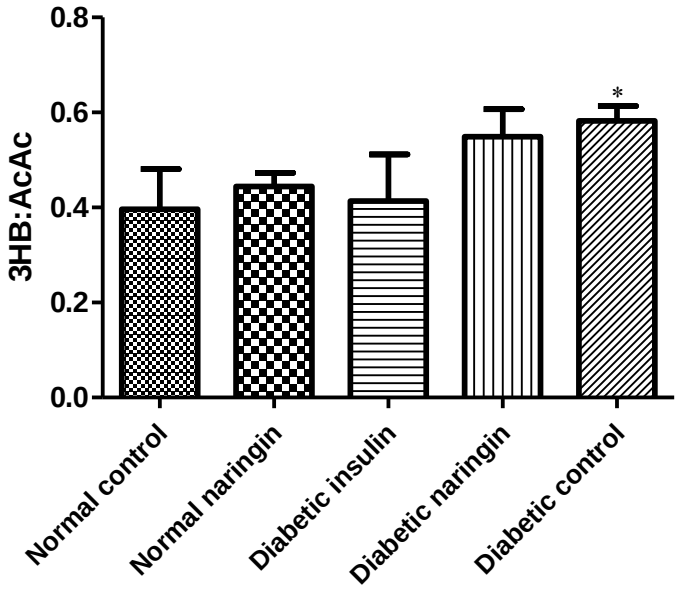

Figure 3.7.D. 3HB:AcAc ratio (*$p<0.05$ compared to normal control).

3.8 Serum electrolyte levels and anion gap (AG)

Serum sodium, potassium and chloride levels did not exhibit any significant differences between the diabetic control and the normal control rat group. Treatment of the diabetic rats with either naringin or insulin also had marginal effects on serum sodium, potassium and chloride ion levels. However, the bicarbonate level of the diabetic control group was significantly ($p<0.0001$) decreased compared to the normal control group. Treatment of the diabetic groups with insulin and naringin significantly ($p<0.0001$ and $p<0.01$ respectively) elevated the bicarbonate concentration compared to the diabetic control group (Table 3.1).

Concentration (mM)	**Normal control**	**Normal naringin**	**Diabetic insulin**	**Diabetic naringin**	**Diabetic control**
Sodium	142.70±0.56	142.80±0.37	140.00±1.00	132.20±1.50	135.00±1.79
Potassium	6.42±0.30	7.120±0.59	5.87±0.12	7.34±0.59	7.56±0.46
Chloride	103.80±0.89	103.00±0.63	100.30±1.33	96.20±2.40	96.40±1.36
Bicarbonate	24.77±0.76	24.74±1.04	21.18±0.69 ***	17.63±0.64 **	13.10±0.54 #

Table 3.1. Serum electrolyte levels (***$p< 0.0001$, **$p< 0.01$ compared to the diabetic control group, #$p<0.0001$ compared to the normal control group).

The AG was significantly ($p<0.0001$) increased in the diabetic control rat group compared to the normal control group. Naringin and insulin treatment respectively, significantly ($p<0.05$) decreased the serum AG in the diabetic groups compared to the diabetic control group (Figure 3.8).

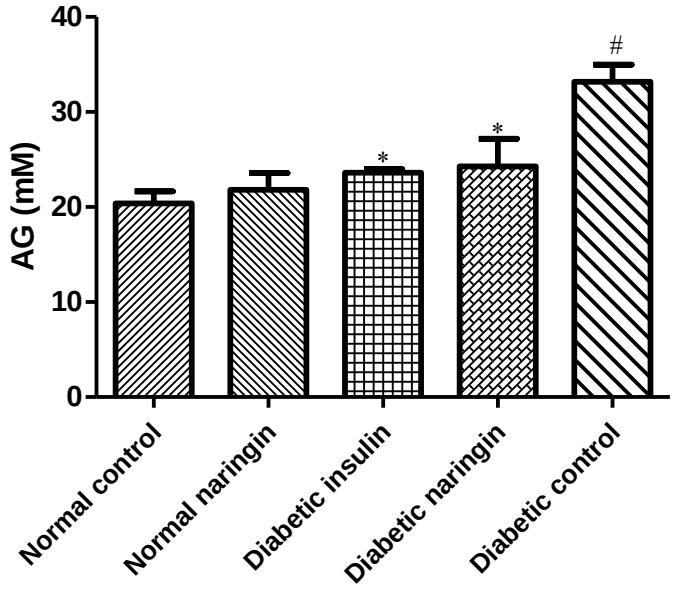

Figure 3.8. Anion gap (AG) (#P< 0.0001, *P< 0.05 compared to the diabetic control group).

3.9 Blood pH levels

The blood pH of the non-treated diabetic group was significantly ($p<0.01$) reduced compared to the normal control group. Treatment of the diabetic groups with naringin and insulin showed significant ($p<0.01$ and $p< 0.05$ respectively) increase in the blood pH compared to the diabetic control group (Table 3.2).

Animal number	**Normal control**	**Normal naringin**	**Diabetic insulin**	**Diabetic naringin**	**Diabetic control**
1	8.5	7.5	8.5	8.5	6.0
2	8.5	9.0	8.0	7.0	6.5
3	7.5	8.0	7.5	7.5	7.0
4	8.5	8.0	8.5	8.5	7.0
5	8.5	8.0	8.0	9.0	6.5
6	7.0	7.5	7.5	8.5	5.5
7	7.5	7.5	7.5	7.0	8.0
Mean	8.00±0.24	7.93±0.20	7.93±0.17*	8.00±0.31**	6.64±0.30#

Table 3.2. Blood pH levels (**$p< 0.01$, *$p< 0.05$ compared to the diabetic control group, #$p< 0.01$ compared to normal control).

Chapter Four

4.0 Discussion

4.1 General

This study investigated the effects of naringin on blood glucose and ketone body homeostasis in DKA in type 1 diabetic Sprague-Dawley rats. The results indicate that naringin does not exert any blood glucose lowering effects in non-diabetic and STZ-induced type 1 diabetic rats. This validates our previous study that naringin does not have blood glucose lowering effects in type 1 diabetes [136]. The results also suggest that naringin treatment does not improve polyuria, AUCs, serum 3HB:AcAc ratio in diabetic rats. However, naringin reversed weight loss, increased hepatic glycogen, decreased serum 3HB, AcAc, TKB and bicarbonate concentrations and improved blood pH and AG in diabetic rats. This suggests that naringin may play a major role in management of DKA.

4.2 Effects of naringin on glucose homeostasis

The diabetic animals used in the study were type 1 diabetic rats with absolute lack of insulin. This was achieved by destroying the pancreatic β-islet cells using STZ, 60 mg/kg body weight. STZ is a glucosamine nitrosourea compound derived from *Streptomyces achromogenes* and used as a chemotherapeutic agent in the treatment of cancer [137]. It selectively destroys the pancreatic β-islet cells. This selectivity has been attributed to the glucose moiety in its structure which enables it to enter the pancreatic β-islet cells using the GLUT-2 glucose transporter [138]. Toxicity to the β-islet cells by STZ occurs as a result of alkylation and damage to the deoxyribonucleic acid (DNA), depleting NAD+ which in turn inhibits insulin secretion and biosynthesis leading to β-cell death through ATP depletion [138]. Another hypothesis cites

damage to DNA through oxidative stress by liberating nitric oxide group from its nitroso moiety [138] which causes the oxidative damage.

The effects of STZ observed in our study included elevation of blood glucose levels, polydipsia, polyuria, weight loss, and hyperketonemia among the STZ-induced diabetic control rats indicating lack of insulin secretion. However, treatment of the normal and diabetic rats with naringin improved the fasting plasma insulin levels suggesting that naringin preserved residual pancreatic β-cell function or induced regeneration of the cells damaged by STZ in the diabetic rats. Weight loss was significantly ($p<0.0001$) elevated in all the diabetic rat groups compared to the normal control rats (Figure 3.1). This is attributed to lack of insulin, an anabolic hormone whose deficiency leads to muscle protein breakdown and decreased synthesis [139]. Furthermore, insulin deficiency is coupled with concomitant increases in insulin counter regulatory hormones namely catecholamines, glucagon, cortisol and growth hormone [58]. These hormones are catabolic in nature and thus contribute to more weight loss. Treatment of the diabetic rats with insulin significantly ($p<0.0001$), (Figure 3.1) improved weight loss suggesting as expected that insulin exerted anabolic metabolic effects. Naringin also significantly ($p<0.0001$), (Figure 3.1) reversed the weight loss. This shows that like insulin, naringin may also be inhibiting lipid and protein catabolism associated with insulin deficiency since naringin treatment on normal non-diabetic rats did not have any significant effect on weight change. This further suggests that naringin does not have any effect on the animal's natural growth.

Polidypsia and polyuria was also observed among the diabetic animals. This was shown by an increased average daily intake of water and urine production respectively that was significantly ($p<0.0001$), (Figure 3.2 and 3.3 respectively) higher in the diabetic groups compared to the normal control group. Hyperglycemia in DKA leads to increased extracellular osmolality which

draws fluid from the intracellular compartment which is then lost through the kidney (diuresis) and may lead to dehydration. As a compensatory mechanism, increased osmolality results in hypothalamic osmoreceptors activating release of a neurohormone, antidiuretic hormone which attempts to correct the hyperosmolar state by acting on the kidneys to conserve water [140] thus decreasing urine output. Failure of this mechanism leads to activation of thirst [140] which leads to the increased water intake. Insulin treatment on the diabetic rats reversed both polyuria and polydipsia. However, naringin treatment on the diabetic rats reversed only polydipsia and had no effect on polyuria. This suggests that naringin inhibited thirst activation but had no anti-diuretic effect in the diabetic state considering the fact that naringin treatment on normal non-diabetic rats did not exhibit any change in urine production when compared to the normal non-treated rats.

Insulin deficiency is associated with increased hepatic gluconeogenesis, decreased glucose utilization, enhanced glycolysis and reduced glycogen synthesis. All these result in hyperglycemia, a key feature characteristic of diabetes. In the study, FBG concentrations were significantly ($p<0.0001$), (Figure 3.4.A) elevated in all the diabetic rat groups compared to the normal control. In addition, calculated AUCs showed that the diabetic groups had significantly ($p<0.0001$), (Figure 3.4.B) increased AUCs compared to the normal control group confirming impairment of glucose tolerance. Surprisingly however, insulin treatment did not improve FBG concentration and AUC compared to the non-treated diabetic rats. Treatment of the diabetic rats with naringin too did not show any significant reduction in blood glucose levels compared to the diabetic control rat group. These observations further support results from a previous study in our laboratory [136] that naringin does not ameliorate hyperglycemia in diabetic type 1 rats. This is despite the fact that numerous studies have reported hypoglycemic effects of naringin and its

aglycone naringenin [133, 141, 142]. The studies [133, 141] reported that naringin acts by suppressing hepatic expression of key gluconeogenic enzymes such as PEPCK and G6Pase. However, majority of these studies have been done in type 2 diabetic animals or diet modified set up. This suggests that naringin exerts metformin-like effects and might require insulin for it to exhibit hypoglycemic effects in diabetes. Metformin, a bi-guanide drug acts by activation of the energy-regulating enzyme, AMP-activated protein kinase (AMPK), to achieve its glycemic control in type 2 DM [143].

The non-treated diabetic rats had significantly ($p<0.0001$), (Figure 3.6) reduced glycogen levels compared to the normal control. This as expected is due to insulin deficiency as insulin improves glycogen storage by increasing expression of glycogen synthase (GS), the rate limiting step in glycogen synthesis [27]. The diabetic groups treated with insulin and naringin significantly ($p<0.05$) (Figure 3.6) increased the hepatic glycogen levels respectively as compared to the non-treated diabetic group. This further supports previous studies which have shown that naringin improves glycogen storage [140, 141], although the studies are conducted in a different metabolic set-up from our study.

4.3 Effects of naringin on ketone body concentration and acid base balance

The levels of 3HB, AcAc and TKB were significantly ($p<0.0001$), (Figures 3.7.A, 3.7.B and 3.7.C respectively) elevated in the diabetic non-treated rat groups as compared to the normal control group showing evidence of ketogenesis. This is expected as a consequence of insulin deficiency which normally inhibits the activity of the enzyme HMGCoA synthase, the rate limiting step in ketogenesis [63]. However, surprisingly, marginal effect was observed on AcAc levels after treatment of the diabetic group with insulin (Figure 15). Like insulin, naringin could

be acting by inhibiting HMGCoA synthase activity as evidenced by decreased serum TKB, AcAc and 3HB levels in comparison to the non-treated diabetic rats. A previous study by Jung *et al.* [133] further showed that naringin reduces the levels of CPT. CPT is involved in the transport of FFA across the mitochondrial membrane into mitochondria for β-oxidation and ketogenesis [61]. Therefore, with reduced CPT levels, there is reduced ketogenesis due to decreased supply of FFA. The ratio of 3HB:AcAc generally reflects the mitochondrial redox state and normally it is usually relatively stable. However, in DKA, there is a reduced mitochondrial redox state which favours 3HB production [50, 63, 64]. This is supported by the results in this study which show a significant ($p<0.05$), (Figure 3.7.D) increase in the ratio among the diabetic rat groups in comparison to the normal control rats. However, treatment with insulin and naringin did not result in any significant improvements in the ratio. The results of the study therefore show that naringin did not cause any shift towards a more reduced state which is more surprising despite its known antioxidant activity.

Increased ketogenesis observed in diabetes is associated with overproduction and accumulation of ketone bodies (AcAc and 3HB) which are strong organic anions. These dissociate freely producing a large amount of hydrogen ions which bind and overwhelm the serum bicarbonate buffering capacity eventually leading to acidosis. This is shown by the decreased bicarbonate concentration and decreased pH among the diabetic animals. However, treatment of the diabetic rats with naringin improved bicarbonate and pH levels significantly ($p<0.05$ and $p<0.01$ respectively), (Table 3.1 and 3.2 respectively) compared to the diabetic control group. Further evidence of an acid base disorder in DKA is seen by the significant ($p<0.05$), (Figure 3.8) increase in AG among the diabetic control group compared to the normal control group. The elevated AG levels in our study further indicate metabolic acidosis. However, this was reversed

by naringin as well as insulin treatment among the diabetic rats with significant ($p<0.01$), (Figure 3.8) improvements observed compared to the diabetic control. These results show that naringin reverses ketogenesis among the diabetic rats. It is also important to note that naringin did not have any significant effects on the ketone body levels in normal rats.

4.4 Conclusion

In this study, Sprague-Dawley rats with type 1 DM showed important biochemical markers for DKA including hyperglycemia, ketonemia, metabolic acidosis, decreased bicarbonate levels coupled with an increased AG which were corrected upon treatment with naringin. The results from this study therefore indicate that naringin as a nutritional supplement could have a protective role against ketoacidosis in type 1 diabetes and might also play a major role as an adjunct therapy in management of patients with DKA but only after conduction of clinical studies. This could be of enormous clinical and economic significance considering the morbidities and mortalities recorded as a result of complications caused by DKA.

Naringin treatment did not improve glucose intolerance in type 1 diabetes. It is therefore likely that it may have been as a result of insulin deficiency. It is therefore suggested that naringin may possess metformin like activity which require insulin to be able exert its anti-hyperglycemic activity. As a result, it is suggested that naringin could also be beneficial in type 2 diabetic patients.

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Appendices

Appendix 1. Animal Ethical Approval for Research

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
Research Office
Animal Ethics Research Committee
Govan Mbeki Centre, Westville Campus,
University Road, Chiltern Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2273/35 Fax (031) 260-2384
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4 September 2013

Reference: 106/13/Animal

Dr P Owira
Discipline of Pharmaceutical
Sciences
School of Health Sciences
WESTVILLE Campus

Dear Dr Owira

RENEWAL: 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 **2012/2013** on the following two-part project:

"1. Potential modulation of the expression and activity of organic cation transporter protein (OCT1), in the rat liver by grapefruit juice and/or naringin in vivo.

2. Effects of grapefruit juice and naringin on serum lipid profile, glucose intolerance and cardiovascular function in diabetic rats."

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc Registrar – Prof. J Meyerowitz
Research Office – Dr N Singh
Head of School – Prof. S Essack
BRU – Dr S Singh

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Appendix 2. Abstract-Diabetic ketoacidosis: an overlooked child killer in sub-Saharan Africa?

Tropical Medicine and International Health doi:10.1111/tmi.1219

VOLUME 18 NO 11 PP 1357–1364 NOVEMBER 2013

Review

Diabetic ketoacidosis: an overlooked child killer in sub-Saharan Africa?

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Summary The true incidence of diabetic ketoacidosis (DKA) in sub-Saharan Africa is unknown but unlike in the Western countries, DKA is also uniquely frequent among type 2 diabetes patients of African origin. Increased hyperglycaemia and hepatic ketogenesis lead to osmotic diuresis, dehydration and tissue hypoxia. Acute complications of DKA include cerebral oedema, which may be compounded by malnutrition, parasitic and microbial infections with rampant tuberculosis and HIV. Overlapping symptoms of these conditions and misdiagnosis of DKA contribute to increased morbidity and mortality. Inability of the patients to afford insulin treatment leads to poor glycemic control as some patients seek alternative treatment from traditional healers or use herbal remedies further complicating the disease process. Standard treatment guidelines for DKA currently used may not be ideal as they are adapted from those of the developed world. Children presenting with suspected DKA should be screened for comorbidities which may complicate fluid and electrolyte replacement therapy protocol. Patient rehabilitation should take into account concurrent treatment for infectious conditions to avoid possible life-threatening drug interactions. We recommend that health systems in sub-Saharan Africa leverage the Expanded Immunization Programme or TB/HIV/AIDS programmes, which are fairly well entrenched to support diabetes services.

keywords diabetes, insulin, ketoacidosis, children, Africa

Appendix 3. **Abstract-College of Health Sciences Research Symposium, 11-12 September 2014.**

NARINGIN IMPROVES KETOACIDOSIS IN TYPE 1 DIABETES

Muranga AN* and Owira PMO*

*Discipline of Pharmaceutical Sciences

Introduction

Naringin, a bioflavonoid is known to possess antidiabetic activity. However, its effects on ketoacidosis, a life threatening complication in diabetes are unknown and were therefore investigated.

Methods

Male Sprague-Dawley rats (225-250g) were divided into 5 groups, (n=7). Group 1 (control) was treated daily with 1.0 ml/kg distilled water, while groups 2 and 4 were treated with 50 mg/kg of naringin, respectively. Diabetes was induced in groups 3, 4 and 5 by a single intraperitoneal injection of 60 mg/kg streptozotocin in 0.1 M citrate buffer and was confirmed after 48 hours. Groups 2 and 3 were further treated with subcutaneous insulin (4 IU/kg) twice daily respectively. Blood samples were collected by cardiac puncture for further analysis.

Results

Group 5 (untreated diabetic) rats exhibited significant ($p<0.001$) weight loss, polydipsia, hyperglycemia, glucose intolerance, low fasting plasma insulin (FPI) and glycogen levels respectively compared to controls. Similarly, they showed significantly elevated plasma malondialdehyde (MDA), beta-hydroxybutyrate ($p<0.001$) and total ketone body (TKB) ($p<0.01$) concentrations compared to the controls. Naringin treatment significantly ($p<0.001$) improved weight loss, FPI and plasma lipid peroxidation respectively while no improvements in polydypsia, hyperglycemia and glucose tolerance levels were recorded compared to the controls. Significant ($p<0.05$) improvements in beta-hydroxybutyrate and acetoacetate levels respectively and TKB ($p<0.01$) were recorded on naringin treatment compared to controls.

Conclusion

Naringin is not hypoglycaemic but improves ketoacidosis in a type 1 diabetes rat model.