UNIVERSITY OF KWAZULU-NATAL

Metformin does not prevent glucose intolerance but improves renal function and reduces oxidative stress in type 1 diabetes

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207503523

2015

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By

CHRISTINE DRIVER

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Submitted in partial fulfilment 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: January 2016

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As the candidate's supervisor, I have approved this dissertation for submission

Signed: Name: Date:

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 September 2014 to November 2015 under the supervision of Dr. Owira P.M.O.

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

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Christine Driver

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Dr Owira P.M.O (Supervisor)

DECLARATIONS

DECLARATION: PLAGIARISM

I, **Christine Driver** 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.

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Signed:

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
AGE	Advanced glycated end-products
AGT	Angiotensin
Akt	Protein kinase β
Ang I	Angiotensin I
Ang II	Angiotensin II
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate activated protein kinase
ATP	Adenosine triphosphate
AUC	Area under the curve
cAMP	Cyclic adenosine monophosphate
DAG	Diacylglycerol
DCCT	Diabetes control and complications trial
DNA	Deoxynucleic acid
ETC	Electron transport chain
FBG	Fasting blood glucose
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GFR	Glomerular filtration rate
GLP-1	Glucagon-like peptide 1
GLUT	Glucose transporter
GSH	Glutathione
GSSG	Glutathione disulphide
GTT	Glucose tolerance test
HbA1c	Heamoglobin A1c
HIV	Human immunodeficiency virus
LA	Lactic acidosis
MDA	Malondialdehyde
mPTP	Mitochondrial permeability pore
NADH	Nicotinamide adenosine dinucleatide
NADPH	Nicotinamide adenosine dinucleotide phosphate
NOX	NADPH oxidase
OCT	Organic cation transporter
PKA	Protein kinase A
PKC	Protein kinase C
PPARS	Peroxisome proliferator activated receptors
RAS	Renin angiotensin system

ROS	Reactive Oxygen Species
SGLT-2	Sodium glucose transporter 2
SOD	Superoxide dismutase
TB	Tuberculosis
TBARS	Thiobarbituric acid reactive substances
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TCA	Tricarboxylic Acid
TBARS	Thiobarbituric acid reactive substances

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ABSTRACT

Type 1 diabetes (T1D) is a chronic condition caused by the complete destruction of insulin producing pancreatic β-cells. Increased oxidative stress and impaired antioxidant capacity are associated with the development of diabetic complications such as diabetic nephropathy. Metformin, a drug commonly used in the treatment of type 2 diabetes, has been suggested to have antioxidant capacity. We hypothesise that metformin, when used as an adjunct to insulin in T1D may help prevent the development of diabetic nephropathy by decreasing oxidative stress.

Sprague-Dawley rats (230-250g) were divided into 5 groups, (Group A: untreated controls, B: diabetic control, C: T1D + insulin (4U/kg twice daily), D: T1D + metformin (250mg/kg via oral gavage), E: T1D + metformin + insulin). Diabetes was induced in groups B-E by intraperitoneal streptozotocin injection at a dose of 65mg/kg body weight and diabetes was confirmed 48 hours later. Glucose tolerance test, serum and urinary electrolytes (K^+,Cl^-,Na^+), creatinine, urea, superoxide dismutase activity, glutathione concentration and malondialdehyde concentration were analysed.

Metformin alone did not improve glucose intolerance. Both the diabetic control group as well as the group treated with metformin alone experienced hyperglycemia, polydipsia, polyuria, weight loss and impaired glucose tolerance. However, when metformin was added to insulin there was a significant increase in electrolyte excretion and also greatly improved creatinine clearance when compared to the diabetic control group. Metformin with insulin further reduced superoxide dismutase activity compared to the diabetic control, increased glutathione concentration as well as reduced malondialdehyde concentrations in both plasma and renal tissue. In conclusion, metformin has positive additive effects on oxidative stress and renal function when used as an adjunct therapy to insulin for T1D.

CHAPTER ONE:
Introduction

1.1 **Diabetes epidemiology**

Diabetes mellitus exists in two forms, type 1 and type 2, which are both characterised by chronic hyperglycaemia. Diabetes occurs when the body either cannot produce insulin or is unable to utilise insulin efficiently or both [1, 2]. In type 1 diabetes hyperglycaemia occurs due to complete destruction of the β- cells in the pancreatic islets of Langerhans. This is usually attributed to autoimmune processes whereby the immune system destroys the beta cells [2, 3]. As a result of the destruction of these cells, there is an absolute deficiency of insulin, leading to complete reliance on exogenous insulin administration for survival [3]. In type 2 diabetes however, insulin effects are diminished due to insulin resistance. This eventually leads to compensatory deficiency in insulin secretion resulting in elevated blood glucose levels [2, 4]. Insulin resistance develops due to alterations in the insulin cell receptor [4]. Factors contributing to the development of type 2 diabetes include: obesity, ethnicity, poor diet and physical inactivity [2].

Rarely, diabetes may be caused by genetic defects, diseases of the pancreas, viral infections and drug/chemical ingestion. Although virus-induced diabetes is rare, congenital rubella, coxsackievirus B, cytomegalovirus, adenovirus and mumps have all been implicated in causing the development of diabetes [4]. Diabetes may also be caused by ingestion of toxins or drugs. Large doses of exogenous steroids such as glucocorticoids can cause impaired insulin action and pancreatitis [4]. Toxins such as pentamidine can cause permanent β-cell destruction, whereas interferon-α treatment has been associated with islet-cell antibodies resulting in insulin deficiency [4].

It is estimated that 8.2% of the global population is diagnosed with some form of diabetes [2]. According to the International Diabetes Federation, 19.8 million Africans had diabetes in 2013 with this figure expected to triple to 66 million by the year 2035 [2]. While South Africa has a 8.27% prevalence rate of diabetes [2]. It was approximated that by the year 2013, 29.7 million Africans were living with impaired glucose tolerance or type 2 diabetes, with 8.3% national prevalence in South Africa [2]. Africa as a whole experiences approximately 6400 new cases of type 1 diabetes annually. South Africa has an incidence rate of 0.8 per 100 000 children between 0-14 years old having type 1 diabetes, suggesting that there is a rapid increase in diabetes cases every year [2]. The reason for the steady increase in diabetes is still not entirely clear, although, environmental factors, diet and viral infections are hypothesised to play a role [2].

Added to the diabetes burden are associated microvascular and macrovascular complications such as nephropathy, retinopathy and cardiovascular disease which result in increased mortality each year. The disease burden of diabetes in Africa is also confounded by the socioeconomic status where a large proportion of diabetic patients go undiagnosed. Thus the true impact of the disease could be much larger than the current statistics suggest [5]. With a high rate of unemployment in South Africa, confirmed cases of diabetes rely on public health funding which is severely constrained by the HIV/TB disease burden [6]. Treatment costs an average diabetic South African $935 annually in diabetes-related expenditure [2]. However, patients have no alternatives as poorly managed diabetes leads to increased morbidity and mortality. These complications, if not actively taken care of, may be debilitating and life expectancy shortening.

The primary aim of treatment for both type 1 diabetes (T1D) and type 2 diabetes (T2D) is optimized glycaemic control which mitigates the development of diabetes complications [7]. T1D is treated using insulin therapy. As insulin is a polypeptide it is orally degraded and therefore is administered normally through subcutaneous injections. However, novel drug delivery systems are currently exploring transdermal and inhaled forms of the drug [8]. Glycaemic control is dependent on patient blood-glucose monitoring and maintaining normoglycemic levels whilst preventing large fluctuations in blood-glucose levels [8]. This has become easier to achieve with the creation of continuous subcutaneous infusion and long-acting insulins [8]. However, these treatments are expensive and are beyond the reach of most diabetic patients. T2D is treated with a combination of lifestyle modifications and drug therapy. Lifestyle modifications include dietary changes, increased exercise and weight control. There are three major groups of oral hypoglycaemic agents used in T2D, these include biguanides, sulphonylureas and thialzolidirediones [1, 8]. Biguanides such as metformin reduce hepatic gluconeogenesis, increase fatty acid oxidation and act as insulin sensitizers. Sulphonylureas are insulin secretagogues as they promote insulin release from the pancreas by blocking ATP-sensitive K^+ channels causing a depolarization, a Ca^{+2} influx and ultimately insulin release [1, 8]. Thialzolidinediones also act as insulin sensitizers by activating peroxisome proliferator-activated receptors (PPARs) and improve insulin sensitivity in the peripheral tissues [1, 8]. Emerging treatments include glucagon-like peptide 1 (GLP-1) agonists, acarbose and sodium-glucose transporter 2 (SGLT-2) inhibitors. GLP-1 agonists are an incretin therapy. GLP-1 receptor agonists enhance insulin secretion and synthesis as well as decrease postprandial glucagon secretion whilst slowing gastric emptying and prolonging satiety [9, 10]. Acarbose prevents the breakdown of complex carbohydrates to monosaccharides whilst reducing postprandial hyperglycemia [10]. SGLT-2 inhibitors increase urinary glucose excretion whilst inhibiting glucose reabsorption [10].

1.2 **Diabetes Complications**

Diabetic complications are largely categorised as microvascular and macrovascular. Microvascular complications include retinopathy, nephropathy and neuropathy while the major macrovascular complications include atherosclerosis leading to accelerated cardiovascular disease, myocardial infarction and cerebrovascular diseases such as strokes [3, 11]. Microvascular complications involve damage to small blood vessels in the body whereas macrovascular complications involve damage to large vessels. [8]

Diabetic retinopathy is associated with damage to retinal vasculature which causes changes in vascular permeability and neovascularisation. This can lead to blindness and visual impairment in diabetic patients [3, 8]. Prolonged hyperglycemia causes changes in the blood-retina barrier [3]. Treatment includes blood pressure and glycaemic control, coupled with invasive procedures such as steroid injections and laser treatment in severe cases [3]

Neuropathy, or nerve damage, is one of the most common long-term complications experienced in diabetes [8]. Diabetic neuropathy involves both the somatic and autonomic peripheral nervous systems and symptoms include loss of sensation, dysesthesia, pain, weakness and impaired wound healing [3, 8]. Neuronal blood flow is compromised by capillary basement membrane thickening causing reduced oxygen supply. Treatment focuses on blood glucose control and pain management[3].

Cardiovascular disease is the leading cause of mortality in T1D [12]. Common cardiovascular disorders in diabetes include hypertension, atherosclerosis, endothelial dysfunction, increased levels of apoptosis in myocytes and an increased risk of myocardial infarction, heart failure and stroke [13]. Patients are managed with blood-pressure lowering agents such as

Angiotensin Converting Enzyme inhibitors (ACE-inhibitors), lipid lowering drugs such as statins and antiplatelet agents such as low-dose aspirin, all whilst maintaining strict glycaemic control using regular anti-diabetic agents [3]

Diabetic nephropathy is one of the most common co-morbidities found in diabetes and contributes significantly to diabetes-related deaths. Diabetic nephropathy encompasses the following functional changes: glomerular hyperfiltration, epithelial hypertrophy, microalbuminuria, glomerular basement membrane thickening and proteinuria [14, 15]. Up to 40% of diabetic patients will develop some form of diabetic nephropathy despite adequate glycaemic control [15]. Current treatment regimens include intensive glycaemic control and the use of ACE-inhibitors. Oxidative stress and prolonged hyperglycemia are the biggest driving forces behind the progression of diabetic nephropathy and other diabetic complications. Therefore, emphasis should be placed on improving the management of these complications as this could decrease the mortality rate associated with diabetes.

1.3 **Impact of hyperglycemia and oxidative stress on diabetic complications**

Hyperglycaemia plays an integral role in the development of diabetic complications. The duration and degree of hyperglycaemia increases the prevalence of complications [16]. It is thought that diabetes complications arise as a result of prolonged periods of hyperglycaemia as well as partially through an increase in oxidative stress and impaired antioxidant capacity [17, 18]. Hyperglycemia causes tissue damage through a number of mechanisms, all of which are as a result of mitochondrial overproduction of Reactive Oxygen Species (ROS) [19]. Hyperglycemia has also been shown to decrease the body's total antioxidant capacity and increase oxidative stress [20]. However, even with well controlled glucose levels, diabetic

patients still develop complications such as nephropathy, retinopathy and cardiovascular disease. This has implicated oxidative stress and antioxidant mechanisms in the development of diabetes complications [21]. The increase in ROS such as superoxide ($O_2^{\bullet-}$) with reduced antioxidant capability is a common phenomenon in diabetes [18]. Superoxide is the initial free radical produced in the mitochondria and activates numerous pathways involved in the pathogenesis of diabetes complications [19].

Hyperglycemia produces oxidative stress and tissue damage through at least 4 recognised mechanisms; increased activity of the polyol pathway, an increase in formation of advance glycated end products (AGEs), increased activation of protein kinase C (PKC) isoforms and overactivity of the hexosamine pathway [19]. In the polyol pathway, excess glucose is converted to sorbitol through activation by aldose reductase which requires Nicotinamide Adenine Dinucleotide Phosphate (NADPH) a major co-factor required to regenerate reduced glutathione (GSH) from its oxidised form (GSSG) [19, 21]. Glutathione is an important scavenger of ROS and its decrease could exacerbate intracellular oxidative stress [19, 21]. In hyperglycaemic conditions, NADPH is used by aldose reductase in the polyol pathway of oxidative stress rather than being used by glutathione in the antioxidant pathway; therefore oxidative stress is preferred and the cell is unable to withstand the oxidative stress insult as the glutathione pool is depleted (Fig. 1) [18].

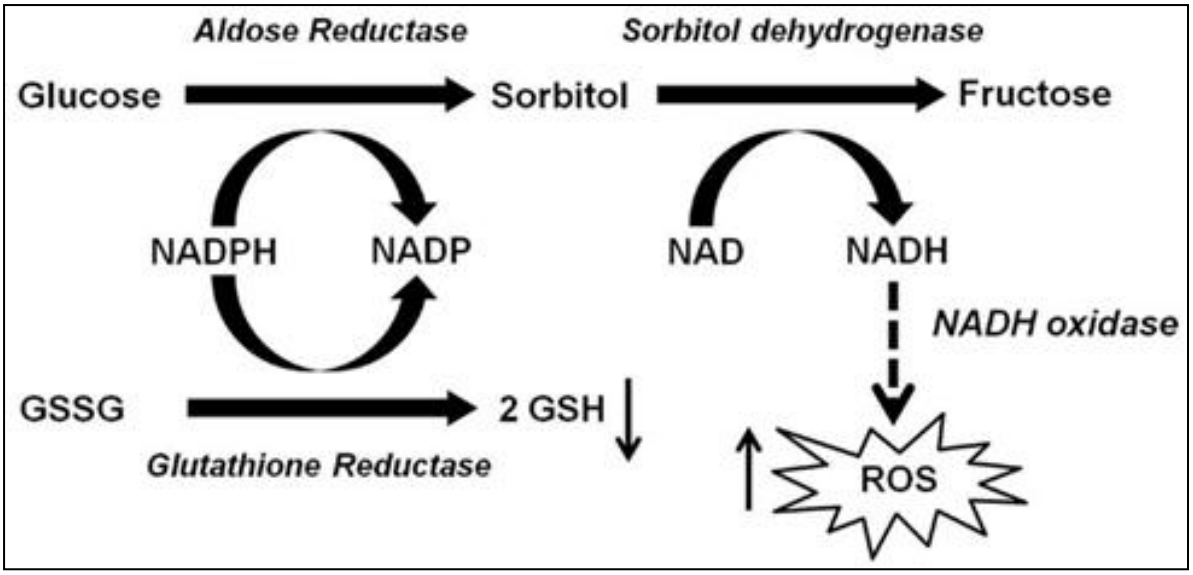

Figure 1: Glutathione depletion due to aldose reductase utilisation of NADPH in polyol pathway [22] .

As a result of increased superoxide production by the mitochondrial Electron Transport Chain (ETC), oxidative stress is increased [19]. Under normal conditions electrons are passed through complexes I-IV and eventually to molecular oxygen which it reduces to water, limiting oxidative phosphorylation (Fig. 2) [19]. However, in diabetic conditions with hyperglycemia present there is a flux of electron donors (NADH and $FADH_2$) to the mitochondrial ETC [21, 23]. This causes the voltage gradient of the mitochondrial membrane to reach critical threshold causing electrons to pile up to coenzyme Q and leak out at complexes I and III [19, 21, 23]. As they are released from the transport chain, the electrons bind molecular oxygen and generate superoxide ions [19, 21, 24]. Under normal conditions electrons reduce molecular oxygen to water; however, under hyperglycaemic conditions oxygen is partially reduced and superoxide is formed instead [21]. Superoxide leads to the inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which ultimately increases the flux into the 4 pathways of hyperglycaemic damage [15, 19, 21]. Reduced activity of GAPDH causes a switch to the polyol pathway, activates PKC, increases AGE

formation and upregulates the hexosamine pathway [21]. For example, inhibition of GAPDH increases intracellular glucose levels which leads to increased activity of the polyol pathway [15, 19]. Similarly when there is reduced GAPDH activity there is an increase in diacylglycerol (DAG) which leads to activation of the PKC pathway [15, 19, 21].

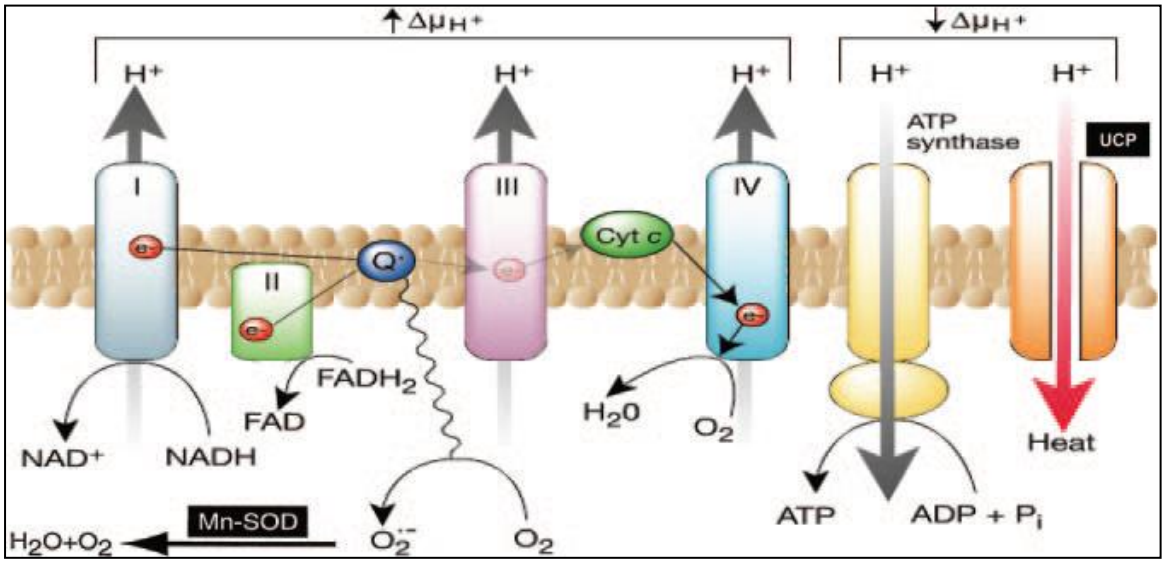

Figure 2: ROS production in electron transport chain [19]

Superoxide dismutase (SOD) is another powerful antioxidant that is capable of combating oxidative stress [3]. SOD converts $O_2^{\bullet-}$ into hydrogen peroxide which is then converted to H_2O and O_2 by catalase [18, 19]. Hence, SOD activity provides a marker for the presence of oxidative stress. It exists in two forms, Cu/Zn-SOD which is found in the cells cytosol and Mn-SOD which is found in the mitochondria [18]. Hyperglycemia has been shown to decrease the concentrations of SOD [20]. It is therefore thought that SOD reduction may perhaps play a role in the development of nephropathy and other diabetes complications [18]. Under hyperglycaemic conditions, ROS causes SOD activity to increase; however, SOD becomes depleted and antioxidant defences compromised, this leads to worsening of diabetes complications such as diabetic nephropathy.

1.4 **Diabetic nephropathy**

Diabetic nephropathy is a common complication both in T1D and T2D [25]. It is estimated that between 20-40% of diabetic patients will develop diabetic nephropathy in their lifetime regardless of glycaemic control [15]. Diabetic nephropathy is a progressive and irreversible renal disease and it is now the leading cause of end-stage renal disease worldwide [25-27]. It is characterised by morphological changes such as extra cellular matrix expansion and thickening of the glomerular basement membrane which lead to kidney damage [25, 27].

Progression of diabetic nephropathy occurs in 5 steps [26]. The first stage, hyperfiltration, is usually seen 3-5 years after the onset of type 1 diabetes and is characterised by increased kidney size, enlarged glomeruli and increased glomerular filtration rate [15, 26]. Stage 2 is silent where glomerular filtration rate returns to normal and there is minimal albumin excretion, it may last 2-5 years [15, 26]. Stage 3 occurs 6-15 years after diagnosis and is associated with basement membrane thickening and extra cellular matrix expansion. Increases in blood pressure may be apparent and glomerular filtration rate (GFR) is either normal or slightly decreased [15, 26]. Stage 4 is overt nephropathy and occurs 10-25 years after diagnosis with type 1 diabetes with visible structural changes, increasing hypertension, and a rapidly falling GFR [15, 26, 28]. At this stage GFR is likely to decrease on average at a rate of 12-15ml/min/year and macroalbuminuria (urinary albumin of between 20-200 mcg/min) may be present [15, 26]. End stage kidney disease is associated with severe hypertension, glomeruloscelrosis and very low GFR and usually occurs 20-30 years after initial diagnosis [15, 26].

Pathogenesis of diabetic nephropathy is not fully understood but 3 major pathways are thought to be involved [25, 29]. Oxidative stress, specifically the formation of AGEs and the

activation of the PKC and polyol pathways, have been proposed to be contributing factors in the development and progression of diabetic nephropathy [15, 27]. Hyperglycemia and increased oxidative stress are the two prominent driving forces associated with the progression of diabetic nephropathy [15].

High glucose concentrations in hyperglycaemic states irreversibly binds with amino acids to form glycosylated products (AGEs) which cause severe damage to the kidney [15]. The AGEs cross-link with collagen and increase the matrix proteins in the glomerular cells. This contributes to the formation of structural changes associated with diabetic nephropathy by increasing basement membrane thickening and exacerbating glomerulosclerosis [15]. AGEs further exacerbate kidney damage by causing increased production of ROS leading to increased oxidative stress [15].

AGEs induce oxidative stress which activates the PKC pathway which plays an important role in the pathogenesis of diabetic nephropathy [25, 27]. Studies suggest that PKC-α activation is involved in the development of albuminuria and PKC-β activation is involved in mesengial expansion, glomerular basement membrane thickening and renal hypertrophy [15]. There is also a link between the polyol pathway and the pathogenesis of diabetic nephropathy where excess glucose is converted to sorbitol through activation by aldose reductase [21]. Studies have shown that when aldose reductase inhibitors are administered, there is decreased urinary albumin excretion suggesting a direct role of aldose reductase in the development of diabetic nephropathy [15].

In diabetic patients, oxidative stress can cause direct damage to the glomerular cells and effect tubule-interstitial structure and function, thus leading to diabetic nephropathy [30].

Patients with diabetic nephropathy exhibit reduced numbers of cells in the kidney which form part of the Bowmans capsule, podocytes. This alters glomerular permeability leading to proteinuria prior to full blown diabetic nephropathy [15, 31, 32]. Podocytopenia is used as a predictor of clinical diabetic nephropathy [29]. Hyperglycemia enhances ROS production in podocytes which concomitantly with NADPH oxidase activation plays a key role in podocyte apoptosis [14, 31]. NADPH oxidase inhibition has been shown to significantly decrease urinary albumin excretion and podocyte depletion [14].

A reduction in renal function may also lead to retention of electrolytes and toxins in the body [33]. It is not uncommon to find hypernatremia and hyperkalemia in the presence of kidney failure [33, 34]. This could potentially lead to additional morbidities such as cardiac dysrhythmias, increased blood pressure and other cardiovascular problems [33, 34].

1.5 **Current treatment of diabetic nephropathy**

Currently, diabetic nephropathy is progressive and irreversible; hence, treatment focus is on delaying disease progression and not on remission [35]. Current strategies to prevent the progression of diabetic nephropathy are multifactorial. These include lifestyle changes, intensive glycaemic control and blood pressure control [36]. Hyperglycemia, hypertension and lifestyle factors such as smoking cause an increase in oxidative stress, inflammation and renal injury [35]. According to the Diabetes Control and Complications Trial (DCCT) intensive glycaemic control (HbA1c of ≤7%) can reduce the risk of new onset microalbuminuria as well as delay the progression of nephropathy [35, 37].

Hypertension is viewed as a high risk factor contributing to both the onset as well as the progression of diabetic nephropathy, therefore blood pressure control is important in delaying

or preventing the development of diabetic nephropathy [36, 38]. Currently type 1 diabetes patients are treated with angiotensin converting enzyme inhibitors (ACE inhibitors) to control hypertension. The renin angiotensin system (RAS) plays an important role in the development of micro- and macrovascular complications such as diabetic nephropathy [39]. Diabetes and hyperglycemia leads to increased RAS activation in tissue and elevated angiotensin II (Ang II) concentrations [40]. Under normal non-diabetic conditions the RAS is stimulated due to hypotension, salt depletion or central nervous system excitation [39]. Prorenin is produced in the juxtaglomerular cells and is cleaved into renin, renin then enzymatically acts on angiotensin (AGT) to form angiotensin I (Ang I), which is catabolised by angiotensin-converting enzyme (ACE) to form the biologically active Ang II [40, 41]. Via the angiotensin type 1 receptor (AT1), Ang II causes vasoconstriction, increased intraglomerular pressure, renal tubule sodium reabsorption, inhibition of renin release, aldosterone secretion and thirst [40]. Aldosterone seceretion is the main cause of sodium and water retention [39]. Ang II has vasoconstrictive effects and causes constriction in the glomerular mesangial cells causing an increase in GFR and leading to kidney damage [41]. ACE inhibitors act on ACE and prevent the conversion of angiotensin I to angiotensin II. ACE inhibitors, through inhibition of the RAS cause relaxation of efferent arterioles, reduction in intraglomerular pressure and proteinuria, therefore leading to delayed progression to End Stage Renal Disease (ESRD) [39]. ACE inhibitor treatment has been shown to prevent an increase in albumin excretion and delay the rate of decline of glomerular filtration rate (GFR) [15, 35]. However, ACE inhibitor treatment is accompanied by its own short-comings and side effects such as the development of a dry cough which is common [39]. ACE inhibitor treatment only slows the progression of diabetic nephropathy and doesn't prevent its development.

A salt restriction diet is also recommended as it is important for blood pressure control and hence, the progression of diabetic nephropathy [35]. Some newer therapies that aim to target ROS directly have been tested yet none has been implemented in the current treatment regimes as clinical trials are still ongoing [35]. Some novel treatments such as aldose reductase targets and NOX-inhibitors are still under review [35]. In recent years it has also been suggested that metformin may possess antioxidant properties and could help prevent or delay the onset of diabetic complications such as diabetic nephropathy and cardiovascular disease.

1.6 **Glucose Metabolism**

Insulin is the gold standard in treatment for type 1 diabetes. The insulin receptor is heterotetrameric and consists of 2 α subunits and 2 β subunits which are linked by disulfide bonds [8, 42]. The extracellular α-subunits bind insulin whilst the β-subunits exist in the transmembrane and possess tyrosine kinase activity [43]. Insulin binding to the α subunit causes transphosphorylation of the β-subunits which leads to an increase in tyrosine kinase activity [42, 43]. Insulin stimulates a decrease in blood glucose concentration by facilitating glucose uptake into the cell by stimulating an increased rate in glucose transporter protein (GLUT-4) translocation from intracellular stores to the plasma membrane in fat and muscle cells [42]. The rate of glucose transport into the cell is dependent on the concentration of GLUT-4 transporters at the cell surface and for how long the concentration is maintained [43]. Insulin increases the rate of translocation of GLUT-4 to the plasma membrane thereby allowing for increased glucose uptake. Following insulin binding, GLUT-4 vesicles move along microtubule tracks before docking and fusing with the plasma membrane allowing for extracellular exposure [42, 43]. Once at the plasma membrane, circulating glucose then passes down a concentration gradient and is diffused into the cell.

Glucose inside the cell is then converted eventually to glycogen and stored after the process of glucose metabolism depending on tissue requirements (Fig. 3).

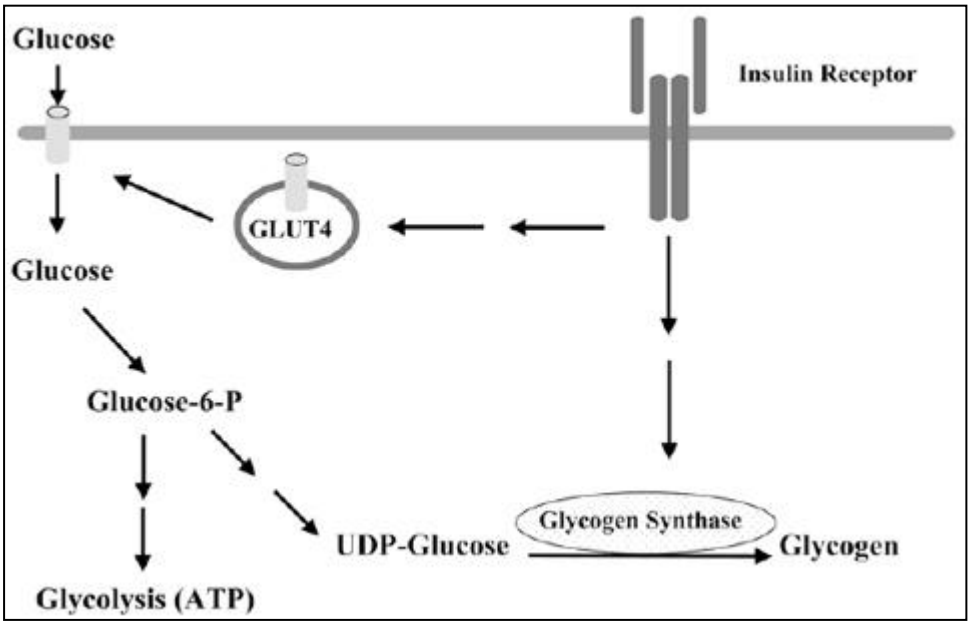

Figure 3: Glucose metabolism pathway [44]

In the presence of insulin, glycogen synthesis is increased while glycogen breakdown is inhibited. Although insulin is not required for glucose uptake by the liver, it stimulates glycogen synthesis and blocks glycogenolysis and gluconeogenesis [42]. Insulin also stimulates the expression of enzymes which favour glycolysis while inhibiting the expression of enzymes which favour gluconeogenesis by stimulating and inhibiting the gene expression which encode for these hepatic enzymes [42]. Insulin activates glycogen synthase by inhibiting kinases such as PKA and protein phosphatase, which downstram decreases the rate of phosphorylation of glycogen synthase, therefore increasing glycogen activity. Circulating blood glucose concentration is therefore decreased after administration of insulin in T1D.

1.7 **Metformin**

Metformin is a popular biguanide drug, commonly used as a first line therapy in the treatment of type 2 diabetes [14]. Metformin is a relatively safe drug. Unlike other glucose lowering agents it very rarely causes hypoglycaemic episodes. It is a well-tolerated drug, with the most common side-effect being gastrointestinal disturbances. Metformin helps reduce blood glucose levels and is referred to as an insulin sensitizer as it increases the effects of insulin [32]. The effects of insulin become more pronounced due to increased insulin sensitivity and is accompanied by a reduction of the amount of insulin required to produce normoglycaemia [45]. Metformin's effects are mediated by its suppression of hepatic gluconeogenesis, decreased fatty acid oxidation and increased peripheral insulin sensitivity, leading to improved glucose uptake in the skeletal muscle and adipocytes [16, 46, 47].

Even though metformin has been in use for the last 50 years, its precise mechanism of action has yet to be elucidated [45, 48]. The liver is the main site of action of metformin [48]. When metformin is administered orally, its highest concentrations can be found in the liver [45, 48]. This is due to the high levels of organic cation transporter 1 (OCT1) expressed in hepatocytes. Metformin is a polar molecule and requires the membrane transporter OCT1 in order to gain entrance into the cell. Once inside the liver, metformin promotes glucose uptake, a decrease in gluconeogenesis, and an increase in glycolysis and glycogen synthesis [48]. It is thought that metformin may work in several ways in order to decrease blood glucose levels. Metformin increases plasma levels of glucagon-like peptide (GLP-1). GLP-1 increases the secretion of insulin and reduces the secretion of glucagon in the presence of glucose [48]. Metformin enhances insulin activity by increasing the translocation of glucose transporter GLUT-2 and GLUT-1 to the plasma membrane within hepatocytes. This results in an increase in the amount of glucose taken up by the liver and consequently increases

glycogen synthesis whilst decreasing gluconeogenesis [48]. Metformin also increases insulin receptor expression [48]. Studies have shown that when metformin is added to insulin therapy in type 1 diabetics there is usually a reduction in the insulin dose required [49, 50]. This is due to metformin's insulin enhancing effects. Less insulin is required in order to achieve normal glycaemic levels. Metformin's major mechanism of action is it's activation of 5'-AMP-activated protein kinase (AMPK) [48, 51].

Once inside the hepatocyte cytosol, metformin acts predominantly in the mitochondria. Although its mechanism is not entirely known, it is thought that through a secondary side effect metformin inhibits complex 1 of the mitochondrial ETC [48]. Among other things this blocks the reverse electron flow at complex 1, common under diabetic conditions, and therefore inhibits mitochondrial ROS production [45]. Metformin has been shown to increase MnSOD levels compared to Cu/ZnSOD. The reason for this is because metformin acts predominantly in the mitochondria and not in the cytosol. MnSOD is found in the mitochondria whereas Cu/ZnSOD is found in the cytosol. Metformin therefore potentially reduces ROS production and increases antioxidant potential [52].

Other studies have suggested that metformin derives the majority of its actions through either direct or indirect activation of AMPK [45, 48, 51]. AMPK is a known master energy sensor, that restores energy balance within the cell [53]. Once activated it switches the cell from an anabolic state to a catabolic state, arresting adenosine triphosphate (ATP)-consuming pathways in favour of ATP-generating pathways. It does this by enhancing catabolic pathways which increase ATP levels such as glycolysis and fatty acid oxidation while inhibiting anabolic pathways that consume ATP [54, 55]. Glycolysis and fatty acid oxidation are the only ATP producing pathways under anaerobic conditions. Under these conditions

glucose, fat and protein synthesis are inhibited [45]. AMPK effects this by phosphorylating 2 protein kinase β (Akt) substrates [55]. Activated AMPK inhibits triglyceride and protein synthesis as well as glucose production in the liver [56, 57].

As a result of metformin transiently inhibiting the mitochondrial electron transport chain, there is a decrease in energy charge and this causes a decrease in ATP production within the cell [45, 48]. The inhibition of complex 1 causes a reduction in NADH oxidation which leads to a lowering of the proton gradient and reduces the proton motive force that is required for ATP production [45, 48]. The decrease in ATP causes a concomitant rise in adenosine monophosphate (AMP) concentration [51]. The increase in AMP is thought to drive two major pathways in metformin's ability to decrease gluconeogenesis. Firstly, elevated AMP inhibits adenylate cyclase which is an enzyme responsible for the conversion of ATP to cAMP. Reduced cAMP has been implicated in a reduction in gluconeogenesis [51]. Suppression of adenylate cyclase in turn down-regulates protein kinase A (PKA) which is a downstream activator of gluconeogenic gene expression [51]. Secondly, an increase in AMP causes an increase in the ratio of AMP:ATP which causes an activation of AMPK [45, 48, 51]. AMPK promotes glucose uptake in the muscles and an inhibition of lipid synthesis in the liver (Fig. 4) [48, 51]. This therefore provides evidence that metformin has an indirect role in the activation of AMPK via complex 1 of the mitochondrial electron transport chain (Fig. 4).

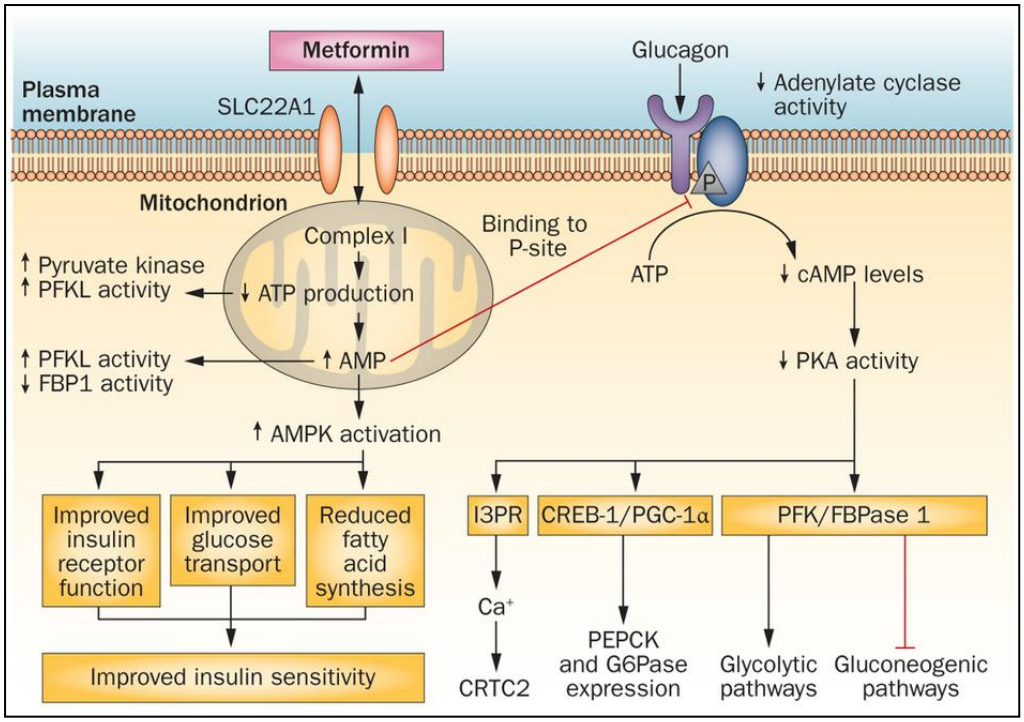

Figure 4: Metformin mechanism of action [48]

1.8 **Metformin use in type 1 diabetes**

There are currently no indications for metformin to be prescribed in T1D. Few studies have looked at the use of metformin in T1D. Of these studies the majority found a significant reduction in the amount of insulin used by patients on a daily basis but found no significant reduction in HbA1c levels [49, 50, 58, 59]. The proposed explanation for this was that patients titrated down their insulin dose in order to maintain their normal glucose levels and to avoid becoming hypoglycemic since metformin increases insulin sensitivity in T1D [59, 60].

It has been well established that metformin treatment in T2D reduces cardiovascular risk factors [60]. Metformin, through its action on eNOS, activates AMPK and reduces vascular inflammation, protects the endothelium from oxidative stress and reduces myocardial injury

after ischemia. By producing glycolytic ATP during periods of cardiac ischemia, AMPK is said to have a cardioprotective role [55]. Through these actions metformin has been shown to decrease the incidence of cardiovascular disease, improve mitochondrial oxygen consumption and improve endothelial function [61, 62]. Some studies have shown a reduction in total cholesterol while on metformin; however, this has not been significant in the majority of studies [49, 60].

The inclusion of metformin with insulin in the treatment of T1D may in theory cause an increase in hypoglycaemic episodes, though the majority of studies found no significant increase in hypoglycaemic episodes [49]. However, this can easily be avoided through glucose monitoring and adjustment of insulin dosage.

After a thorough literature review it was found that there have been no previous studies evaluating metformin effects on diabetic nephropathy in T1D.

1.9 **Metformin use in chronic kidney disease**

Although metformin is used as a first-line treatment of type 2 diabetes (T2D), there are concerns of its safety in patients with kidney disease. This is because it is excreted almost exclusively via the renal system with a potential of causing lactic acidosis. Lactic acidosis is a serious condition where arterial lactate levels are elevated and blood pH drops to below 7.35 [63]. Another biguanide, metformin's predecessor, phenformin was removed from the market due to a high incidence of drug induced lactic acidosis (LA) [64]. Though the risk of lactic acidosis on metformin has been shown to be relatively low, bigaunides have been shown to increase plasma lactate concentrations and concurrently decrease lactate metabolism [65]. Phenformin induces the conversion of glucose to lactate and impairs its own

renal excretion [66]. It causes lactic acidosis through pyruvate accumulation due to gluconeogenesis inhibition. Oxidation of fatty acids causes a depletion of NAD^+ and increases NADH. Simultaneously, phenformin inhibition of oxidative phosphorylation impairs NAD^+ generation from NADH which further increases the NADH/NAD^+ ratio. A increase in this ratio inhibits pyruvate dehydrogenase and therefore inhibits pyruvate entry into the Krebs cycle [65]. This causes an accumulation of pyruvate which is then metabolised to lactate. Fatty acid metabolism which produces ketone bodies, cause further exacerbation of acidosis [65]. Biguanide-associated lactic acidosis has also been linked to their inhibition of complex 1 of the mitochondrial respiratory chain, where inhibition of mitochondrial oxidative phosphorylation is correlated with increased plasma lactate concentration [63].

Reports suggest that metformin has an incidence rate of LA 20 times less than phenformin [63]. Metformin has a wider therapeutic window compared to phenformin and requires higher blood concentrations of the drug to produce lactic acidosis [63]. Phenformin undergoes hepatic metabolism and is excreted renally whereas metformin is excreted unchanged by the kidneys [65-67]. Therapeutic doses of metformin causes almost no increase in blood lactate levels [63] Due to perceived risk of LA in renal impairment, most guidelines recommended that when glomerular filtration drops to <60 ml/min, metformin therapy should be discontinued [64]. However, this has recently been revised to discontinuation when GFR is <30 ml/min [64]. Studies have shown that there is no difference in the incidence of LA in diabetic patients taking metformin as opposed to those not on metformin [67]. Another study found no difference in the incidence of lactic acidosis between metformin and sulfonylureas [63].

Metformin not only improves glycaemic control but also has cardioprotective properties and has been proposed to have renoprotective effects which could potentially delay the onset of

nephropathy [68]. Therefore, the benefits of metformin therapy could outweigh the risks, even in chronic kidney disease.

1.10 Metformin use to prevent diabetic nephropathy

Although the exact mechanism of action by which metformin could exert renoprotective effects is unknown, the ability of metformin to reduce ROS as well as prevent tubular cell injury may be the contributing factor [29, 32, 69]. Morales et al. (2010) showed that metformin can improve gentamycin induced nephrotoxicity and renal tubular injury [70]. Gentamycin is a common nephrotoxic antibiotic and causes necrosis of the proximal tubular cells, apoptosis and reduced levels of ATP in the renal tubular cells [70]. Metformin was shown to improve renal blood flow as well as prevent mitochondrion derived oxidative stress, possibly through its transient activity at complex-1 on the respiratory electron chain [70]. In the same study, it was shown that metformin has preconditioning effects by preventing the opening of the mitochondrial permeability pore (mPTP). Opening of the mPTP causes an influx of solutes and water, this causes the outer mitochondrial membrane to rupture and release the contents of the inter-membranous space into the cytosol [71]. Pro-apoptotic factors are released which lead to necrosis, apoptosis and cell death [71, 72].

In a diabetic state metformin has been reported to have a protective effect on the renal tubules and podocytes [31, 32]. As with gentamycin induced toxicity, it is thought that ROS produced in the mitochondria may play a significant role in diabetic nephropathy [32]. Pro-apoptotic factors which are released may cause cell damage, there is therefore merit in attempting to protect the mitochondria and prevent mitochondrial ROS production to prevent nephrotoxicity [32]. Based on these findings it is conceivable to suggest that metformin can attenuate diabetic nephropathy by decreasing ROS production and oxidative injury [32].

Additionally metformin not only has protective effects but also has ameliorative properties, and is capable of reversing renal damage [32]. While ROS levels are elevated in a diabetic conditions, AMPK levels are reduced [73, 74] suggesting that hyperglycaemia decreases antioxidant capacity [17, 18]

It has been shown in diabetic rats that podocyte density is decreased with increased albuminuria which is attenuated with metformin therapy suggesting a reversal of podocyte injury [32, 73]. It is hypothesised that metformin's renoprotective effects could at least be partly due to its anti-oxidative activity[73]. The most likely mechanism of action for this would either be through metformin's inhibition of complex 1 of the mitochondrial electron transport chain, and/or its activation of AMPK [31, 73].

As metformin is a well-tolerated, safe drug which may possess positive antioxidant abilities, this study therefore seeks to investigate the merits of using metformin as an adjunctive therapy to insulin in the treatment of T1D in order to prevent or delay the occurrence of diabetes associated complications, namely diabetic nephropathy.

1.11 **Aim**

To investigate the effects of metformin on renal function and glucose tolerance in a type 1 diabetes rat model.

1.12 **Objectives**

- To investigate metformin's effects on glycaemic control and glucose tolerance in T1D
- To investigate metformin's effect on oxidative stress in plasma and renal tissue and its effect on aspects of renal function

CHAPTER TWO:

MATERIALS AND METHODS

2.1 **Chemical Reagents**

The following reagents were purchased from Sigma-Aldrich Pty. Ltd, Johannesburg South Africa; streptozotocin, D-glucose, citrate buffer, phosphate buffer, thiobarbituric acid, metaphosphoric acid chips, butylated hydroxytoluene and phosphoric acid.

Metformin (Accord, Sandton, South Africa), insulin (Novo Nordisk, Norway), portable glucometers and glucose test strips (OneTouch Select, Zug, Switzerland) were purchased from a local pharmacy.

A superoxide dismutase assay kit and a glutathione assay kit were purchased from Cayman Chemicals (Michigan, USA). Insulin ELISA test kit was purchased from Mercodia Chemicals (Uppsala, Sweden). Halothane used to euthanize the animals were provided by the Biomedical Resourse Unit (BRU) of the University of KwaZulu-Natal, Durban, South Africa.

2.2 **Animal Treatment**

Male Sprague-Dawley rats weighing between 230-300 g were used in the study. Animals were housed at 7 rats per cage and had free access to food and drinking water for the duration of the study (56 days). The rats were divided into 5 groups (n =7). The rats were maintained on a 12-hour dark/light cycle (08:00-20:00) in an air-controlled room (temperature 25°C +/- 2°C, humidity 55 +/- 5%). Rats were fed

normal chow containing; protein, fibre, starch and calcium. All animals completed the study.

2.3 **Ethics**

Ethics approval was obtained from the Animal Research Ethics Committee of The University of KwaZulu-Natal. Ethics reference number: 078/14/Animal.

2.4 **Induction of Experimental Diabetes**

Diabetes was induced in groups B-E. Fasting glucose levels were taken prior to induction to ensure rats were not already diabetic. Diabetes was induced via a single intraperitoneal injection of streptozotocin (65 mg/kg body weight) from a stock solution at a concentration of 36 mg/ml. Streptozotocin was dissolved in 0.1 M citrate buffer of pH 4.5. Citrate buffer was made using 10 mM of sodium citrate and 10 mM citric acid. Induction took place after overnight fasting. Diabetes was confirmed 48 hours after the administration of streptozotocin solution. Confirmation of diabetes diagnosis was carried out via fasting blood glucose tests. Blood was obtained via tail pricks and then blood glucose levels tested using glucostrips with a hand-held glucometer (Bayer Acensia). Rats with fasting blood glucose of 7.5 mmol/l and above were considered diabetic.

2.5 **Experimental Design**

Rats were divided into the following groups named A-E. Groups B-E were made type 1 diabetic through intraperitoneal streptozotocin (STZ) injection as previous explained.

	Treatment			STZ 65mg/kg body weight (bw)
Groups	Distilled water (1ml/kg bw)	Insulin (4 U/kg bw, bd via susubcutaneous injection)	Metformin ((250mg/kg) once daily, via oral gavage)	
A – Normal control	✓			
B- Diabetic Control	✓			✓
C – Insulin		✓		✓
D- Metformin			✓	✓
E-Metformin + Ins		✓	✓	✓

Table 1: Animal treatment schedule

Rats were housed 7 per cage and had free access to food and water for the duration of the study. Bedding was changed daily. The rats were weighed daily and the weights obtained were recorded. The amount of water consumed was also measured daily and recorded. Random fasting blood glucose tests were carried out every 14 days with the results also being recorded. On day 51 of the study the rats were moved into metabolic cages for a period of 24 hours. Throughout this time urine was collected in a calibrated container attached to the cage. At the end of the 24 hours the urine collected was measured and then decanted into appropriate test containers before being stored at -80 °C for further analysis. On day 54 a glucose tolerance test was performed.

On day 56 the animals were euthanized via halothane overdose. Blood samples were obtained via cardiac puncture and stored in appropriate test tubes. A portion of blood sample was placed in heparin test tubes while another portion placed in plain uncoated test tubes. Both tubes were centrifuged and the supernatant, either plasma or serum, was aliquoted out and placed in Eppendorf tubes. They were then stored at -80 °C until further analysis. The kidney was quickly excised. The organs were washed in phosphate-buffered saline before being snap-frozen in liquid nitrogen and stored at -80 °C until further analysis.

2.6 **Glucose Tolerance Tests**

On day 54 of the study, a glucose tolerance test was carried out. Before the GTT all animals were starved overnight and fasting blood glucose (FBG) concentrations were determined on the morning of the test before glucose administration. Rats received an intraperitoneal injection of 3.0 g/kg body weight of glucose in normal saline. Blood

glucose concentrations were then taken at 15, 30, 60, 90 and 120 minutes, respectively in all treatment groups.

Area under the curve (AUC) was calculated from a blood glucose vs time plot and expressed as mmol/l x time (min), (AUC units).

2.7 **Electrolytes**

Electrolyte concentrations were determined in both serum and urine samples using Beckman Coulter Synchron Aqua CAL system for chloride, potassium and sodium diagnostic kits. For each sample, a volume of 40 µl sample was mixed with 1.32 ml of buffer solution (provided in diagnostic kit) in a ratio of 1 part sample to 33 parts buffer. The concentrations of the various electrolytes were determined using indirect potentiometry. In the determination of potassium and chloride ions a potassium ion selective electrode or a solid state chloride electrode was used respectively in conjunction with a sodium reference electrode. In the determination of sodium ion concentration, 2 glass sodium electrodes were used, with one acting as a reference. Electrolyte buffer reagent as well as electrolyte reference reagents were provided in the diagnostic kits. The electrolyte buffer reagent was constituted with Tris buffer.

2.8 **Creatinine Concentrations**

Creatinine concentrations were determined in urine and serum sample by a Beckman Coulter diagnostic kit. The system utilised a Jaffe method to determine the concentrations of the creatinine. Serum sample was combined with reagent solution in a ratio of 1:11 that is 20 µl of serum with 220 µl of reagent [75]. Urine samples were combined with reagent in a ratio of 1:73 that is 3 µl urine with 219 µl of reagent. The creatinine in the sample combined with picrate in an alkaline solution to form a deep

red creatinine-picrate complex. The colourimetric reaction was proportional to the amount of creatinine in the sample. The change in absorbance at 520 nm was measured. This absorbance was proportional to the concentration of creatinine in the sample.

2.9 **Creatinine Clearance**

Creatinine clearance was calculated using the following formula:

$$CrCl = \frac{Creatinine\ urine\ (umol/l)}{Creatinine\ serum\ (umol/l)}\ x\ \frac{urine\ volume\ (ml)}{time\ (\text{min})}$$

2.10 **Urea**

Urea concentrations were determined in urine samples by a Beckman Coulter diagnostic kit. An enzymatic rate reaction is used to determine the concentration of the urea in the urine samples. The reaction is illustrated by the following chemical equations:

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \alpha\text{-Ketoglutarate} + NADH + H^+ \xrightarrow{GLDH} \text{Glutamate} + NAD^+ + H_20$$

The urine samples were diluted in a ratio of 1 part urine to 100 parts reagent (3 µl urine to 300 µl of reagent). The change of absorbance was measured by at 340 nm and was proportional to the concentrations of urea in the samples.

2.11 **Plasma Insulin**

An ultrasensitive rat insulin ELISA kit was used to determine the plasma insulin levels in the samples as per the manufacturer's instruction using a 96-well microtitre plate. In brief, 25 µl of calibrator and samples were pipetted into each well before 100 µl of enzyme conjugate 1X solution was added. The plate was then incubated on a plate shaker (700-900 rpm) at room temperature (25°C) for 2 hours. The reaction volume was then

discarded before the plate was washed manually 6 times using 350 µl of the provided wash solution in each well. Thereafter, 200 µl of substrate tetramethylbenzidine (TMB) was added into each well before being incubated at room temperature for 15 minutes, after which 50 µl of stop solution was placed in each well. The plate was then placed on a plate shaker for approximately 5 seconds to ensure mixing. Optical density was read on a microplate reader (Biochron, EZ Read 400) at 450 nm and the results calculated. A calibration curve was drawn from the standard samples provided and the insulin plasma concentration of the test samples were read off the calibration curve via interpolation.

2.12 **Superoxide Dismutase (SOD) Assay**

Both manganese superoxide dismutase (MnSOD) and copper/zinc superoxide dismutase (CuSOD) were measured in kidney tissue samples. A SOD assay kit was used as per the manufacturer's instructions using a 96-well microtitre plate. Kidney tissues were prepared by homogenisation 1:7 w/v in ice cold buffer, pH 7.2 (containing 1mM EGTA, 210mM mannitol, 70nM sucrose and 20mM HEPES). The samples of homogenised tissue in buffer were then centrifuged at 1500 xg for 5 minutes at 4 °C before being stored at -80 °C for further analysis.

In brief, the assay was performed by adding 200 µl Radical detector (tetrazolium salt solution) and 10 µl of sample or standard into the well before initiating the reaction by adding 20 µl of diluted xanthine oxidase to all the wells. When performing the assay for MnSOD, 190 µl of radical detector and 10 µl of 3 mM potassium cyanide were added along with the sample and xanthine oxidase. The plate was then covered and incubated at room temperature on a plate shaker for 30 minutes. Absorbance was read at 450 nm using a microplate reader (Biochron, EZ Read 400) and the results calculated. The linearised rate (LR) of the standards

and samples were calculated by dividing standard A's absorbance by all the other standards and samples. The linearised SOD standard rate was then plotted as a function of SOD activity (given) in order to produce a standard curve. The standard curve produced an equation which was used to calculate the SOD activity of the samples:

$$SOD\ \left(\frac{U}{ml}\right) = \left[\frac{sample\ LR - y\ interecpt}{slope} \times \frac{0.23ml}{0.01ml}\right] \times sample\ dilution$$

Where LR is Linearised Rate

Reagents such as radical detector, xanthine oxidase and standards were provided by the kit and prepared by using the manufacturer's instructions.

2.13 **Glutathione Concentrations**

A glutathione assay kit was used to determine the total glutathione concentrations in kidney homogenates as per the manufacturer's instructions using a 96-well microtitre plate. Kidney tissues were prepared by homogenisation 1:7 w/v in cold buffer, pH 7 (containing 50 mM MES and 1 mM EDTA). The samples were then centrifuged at 10 000 xg for 15 minutes at 4°C before the supernatant was removed and stored at -20°C in preparation for deproteination.

For deproteination an equal volume of sample was mixed with metaphosphoric acid solution by vortexing. It was then centrifuged at 2000 xg for 2 minutes before the supernatant was collected. A solution of 4 M triethanolamine was prepared. This solution was then mixed with the supernatant in a ratio of 50 µl triethanolamine solution per 1ml supernatant.

The assay was carried out by placing 50 µl of sample and standards into designated wells, then adding150 µl of Assay Cocktail (prepared using the manufacturer's instructions) to each well before incubating in the dark on an orbital shaker and measuring the absorbance at 410

nm. Glutathione concentrations were then calculated by plotting a standard curve of glutathione concentration versus absorbance. The sample concentrations were the calculated through interpolation on the standard curve.

2.14 **Lipid Peroxidation - Plasma TBARS Assay**

Lipid peroxidation was quantified by measuring the concentrations of malondialdehyde (MDA) in plasma samples obtained from treatment animals. The assay was carried out according to the modified method of Phulukdaree et al [76]. In brief, a TBA/BHT solution was prepared using thiobarbituric acid (TBA) (1% w/v)/ 0.1 mM butylated hydroxytoluene (BHT).

In a set of clean tubes 200 ul of samples were added to a solution of 500 µl of 2% phosphoric acid (H_3PO_4), 400µl of 7% H_3PO_4, 400µl of the TBA/BHT and 200µl of 1 M HCl respcetively. The tubes were incubated in boiling water (100°C) for 15 minutes before being cooled to room temperature. Once cooled, 1.5 ml of butanol was then added to each test tube and vortexed. Following vortexing, 200 µl of the top phase was transferred into a 96-well microtitre plate and read at 532 nm and 600 nm respectively using a microplate reader (Biochron, EZ Read 400). The plasma malondialedhyde (MDA) concentrations were then determined by using extinction coefficient 156 mM^{-1}.

2.15 **Lipid Peroxidation - Renal Tissue TBARS Assay**

Tissue TBARS assay was carried out according to the modified method of Hermes-Lima et al [77]. Frozen kidney tissue was homogenised in a 1:10 (w/v) ratio with cold 1.1% phosphoric acid. A solution containing 1% TBA, 50 mM NaOH and 0.1 mM BHT was made for the assay. In a clean set of test tubes 400 µl of sample homogenate was added to 400 µl

TBA/BHT solution and 200 µl of 7% phosphoric acid (resulting in a final pH of 1.6). Test tubes were then incubated in a boiling water bath for 15 minutes before being cooled to room temperature. Once cooled, 1.5 ml of butanol was then added to each test tube and vortexed. Following vortexing the solution splits into 2 phases, 200 µl of the top phase from each test tube was transferred into a 96-well microtitre plate and read at 532 nm and 600 nm respectively using a microplate reader (Biochron, EZ Read 400). The plasma MDA concentrations were then determined by using extinction coefficient 156 mM^{-1}.

2.16 **Statistical Significance**

Data was presented as mean ± SD and analyzed using the statistical software, GraphPad Prism (San Diego, USA) version 5.0. Student t-tests or 1-way ANOVA were carried out where appropriate. A p-value of <0.005 was considered statistically significant.

CHAPTER THREE:

RESULTS

3.1 NATURAL GROWTH MEASURED BY WEIGHT GAIN

Animals were weighed daily throughout the study period. These weights were plotted against time to show the growth trend.

Untreated diabetic control animals experienced negative weight gain compared to the normal controls (Fig. 5). However, treatment with insulin or a combination of insulin + metformin resulted in steady positive weight gain. Metformin treatment alone did not improve weight loss compared to the untreated diabetic controls.

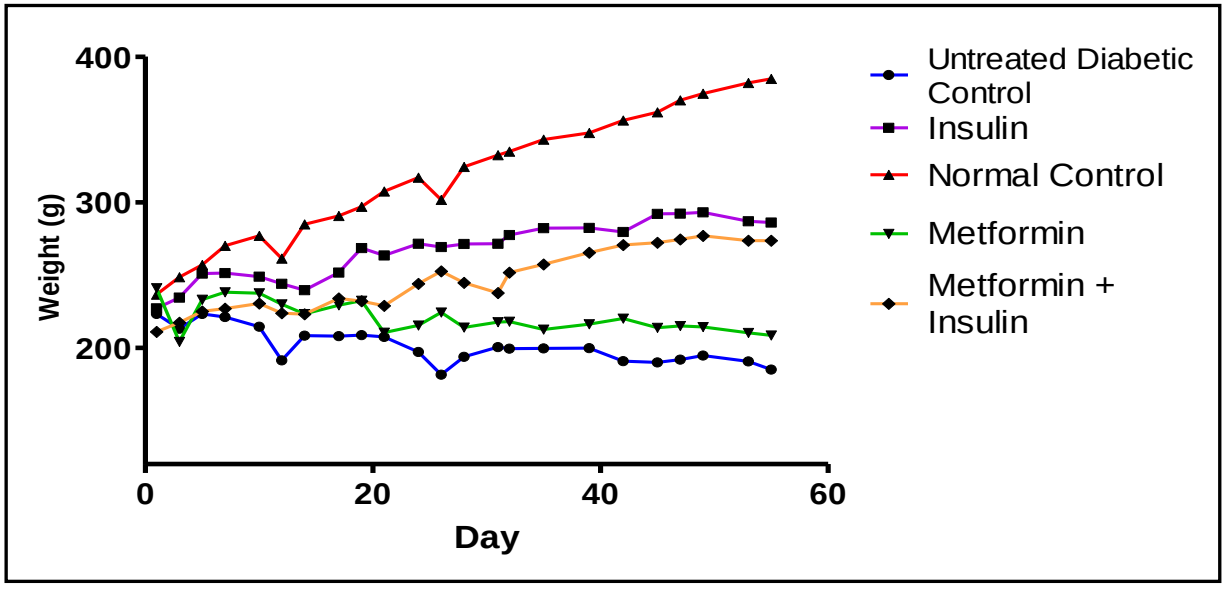

Figure 5: Animal live weights over time. Weight changes during treatment period for the different treatment groups

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The difference between final weight and initial weight was calculated per treatment group. The average change in weight between the normal control and the untreated diabetic control groups was highly significant ($p < 0.0001$) (Fig. 6). Both the insulin and the metformin + insulin group had significantly ($p<0.0001$) increased weight compared to the untreated diabetic control group (Fig. 6). However, no significant difference occurred between the untreated diabetic control group and the group treated with metformin alone.

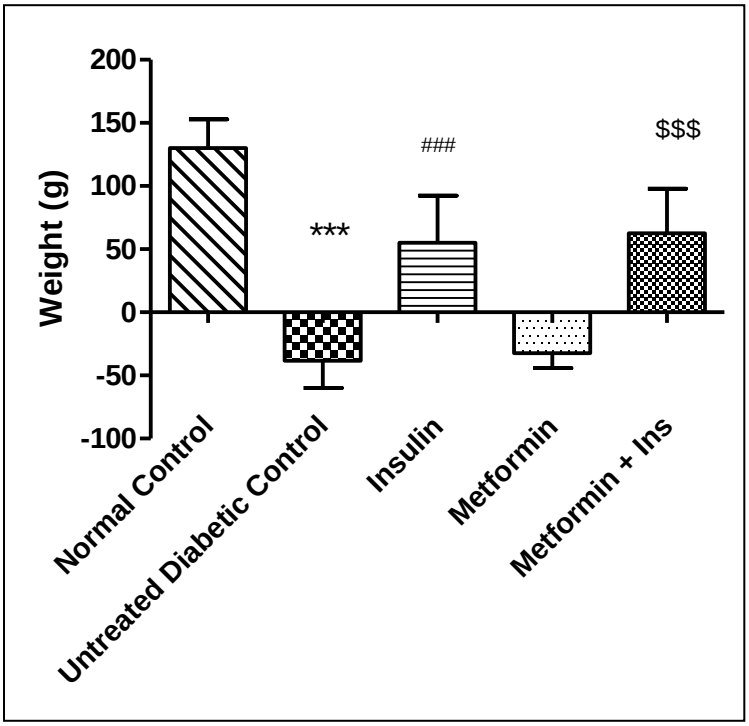

Figure 6: Changes in animal live weights. Animals were weighed at the beginning and at the end of the study and the differences in weights calculated. *** $p < 0.0001$ compared to normal control. ### $p < 0.05$ compared to untreated diabetic control. $$$ $p< 0.0001$ compared to untreated diabetic control.

3.2 **WATER CONSUMPTION**

Water consumption was measured daily and the average amount per animal in each treatment group per day was calculated.

Untreated diabetic animals consumed significantly ($p<0.0001$) more water compared to the normal controls suggesting increased polydipsia. Treatment with insulin, with or without metformin reduced the amount of water consumed by the animals compared to the untreated diabetic control, however this was not significant (Fig. 7).

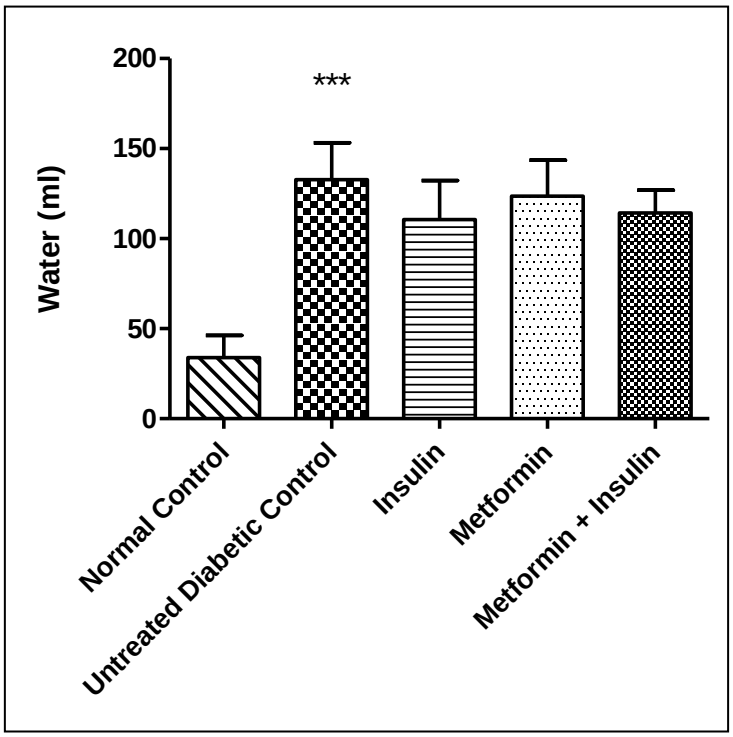

Figure 7: Average Daily Water Consumption in Designated Treatment Groups, calculated by taking the total volume drunk by the group and dividing by the total number of rats in the cage. *** $p < 0.0001$ compared to normal control group.

3.3 **URINE OUTPUT**

Untreated diabetic controls produced significantly more urine over 24 hrs than the normal controls ($p < 0.001$). Insulin with or without metformin insignificantly reduced urine output compared to untreated diabetic controls (Fig. 8).

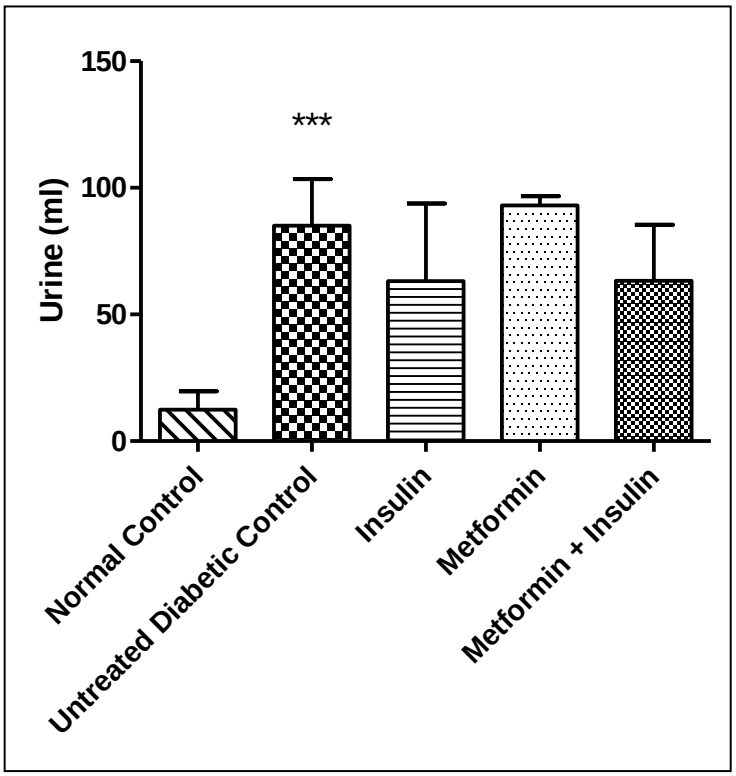

Figure 8: Average 24 hour urine output. Output was measured by placing the test animals in metabolic cages for 24 hours. Metabolic cages had plastic vessels attached to specifically collect the urine. *** $p<0.0001$ compared to normal control.

3.4 **FASTING BLOOD GLUCOSE**

Diabetic animals had significantly ($p < 0.0001$) higher fasting blood glucose levels compared to the normal controls. Blood glucose levels for the diabetic controls as well as the metformin group, increased as the study progressed. Treatment with metformin or insulin did not significantly improve FBG compared to the untreated diabetic rats.

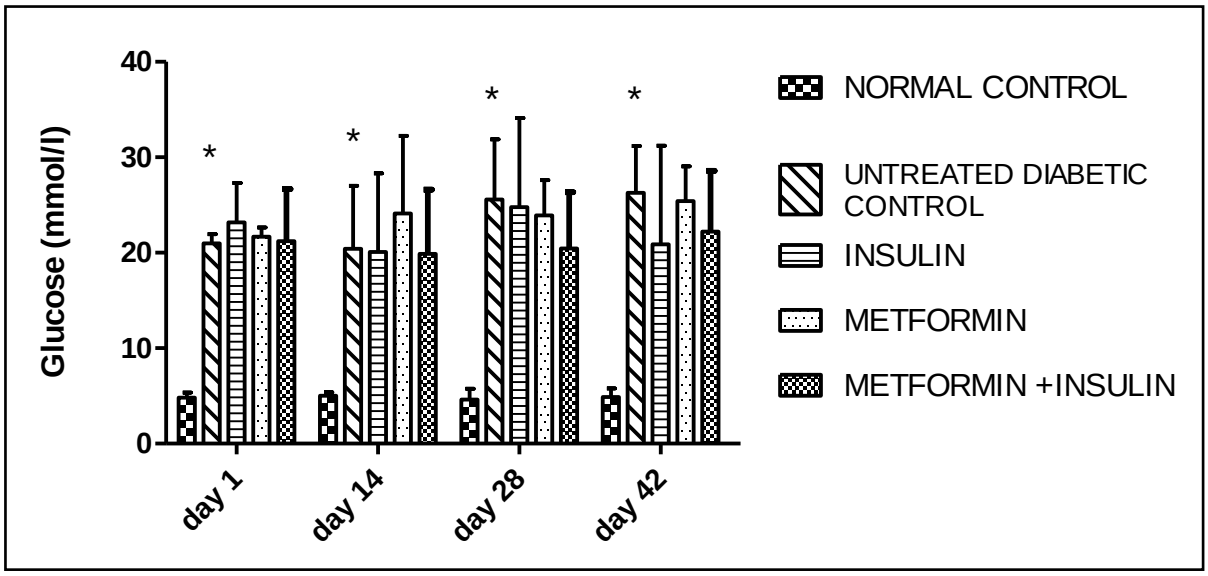

Figure 9: Fasting blood glucose concentrations measured in each designated group. Blood was obtained via tail prick and glucose levels measured using a hand held glucometer. *$p<0.0001$ for untreated control versus untreated diabetic control.

3.5 **GLUCOSE TOLERANCE TEST**

Diabetic animals had significantly higher ($p < 0.0001$) fasting blood glucose levels compared to normal controls (Fig. 10a). Untreated diabetic controls and the metformin group had high basal glucose levels compared to the normal control. Following the intraperitoneal injection of glucose, all groups experienced an initial spike in blood glucose levels but blood glucose concentrations only returned to baseline after 2 hours in normal controls (Fig. 10a).

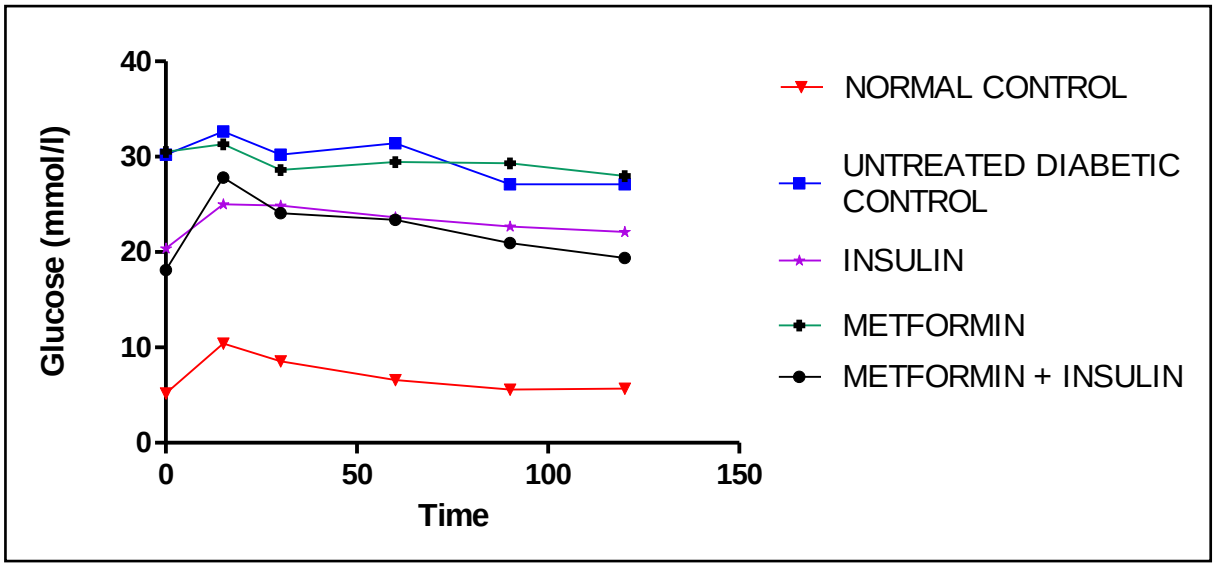

Figure 10a: Glucose Tolerance Tests. Blood glucose concentrations plotted against time after the animals were injected intraperitoneally with glucose (3.0g/kg) in normal saline, blood glucose levels were measured at specific time intervals over a period of 2 hours.

Calculated AUC for the untreated diabetic controls was significantly ($p < 0.0001$) higher compared to the normal controls ($p<0.0001$). Treatment with metformin + insulin resulted in significantly ($p < 0.05$) reduced AUC when compared to untreated diabetic rats ($p<0.05$) (Fig. 10b). Insulin also reduced AUC compared to untreated diabetic controls, however this was insignificant. Treatment with metformin alone resulted in high AUC levels and had no statistically significant difference compared to untreated diabetic controls.

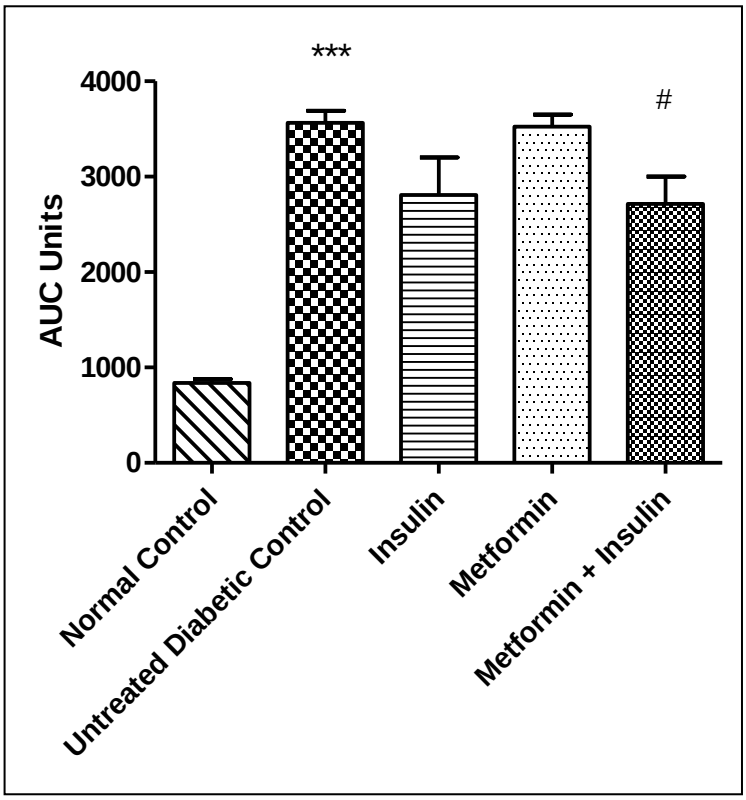

Figure 10b: Calculated area under the curve (AUC) from blood glucose vs time plots. AUC was measured as mmol/l x time to give AUC units. *** $p< 0.0001$ in two-tailed t-test for control group versus diabetic control group. # $p < 0.05$ in a two –tailed t-test for metformin+ insulin versus untreated diabetic control

3.6 **FASTING PLASMA INSULIN**

Untreated diabetic rats exhibited significantly ($p<0.0001$) lower plasma insulin levels compared to normal controls (Fig. 11). However, treatment with insulin or metformin + insulin resulted in significantly ($p<0.05$) elevated plasma insulin levels compared to untreated diabetic rats (Fig. 11).

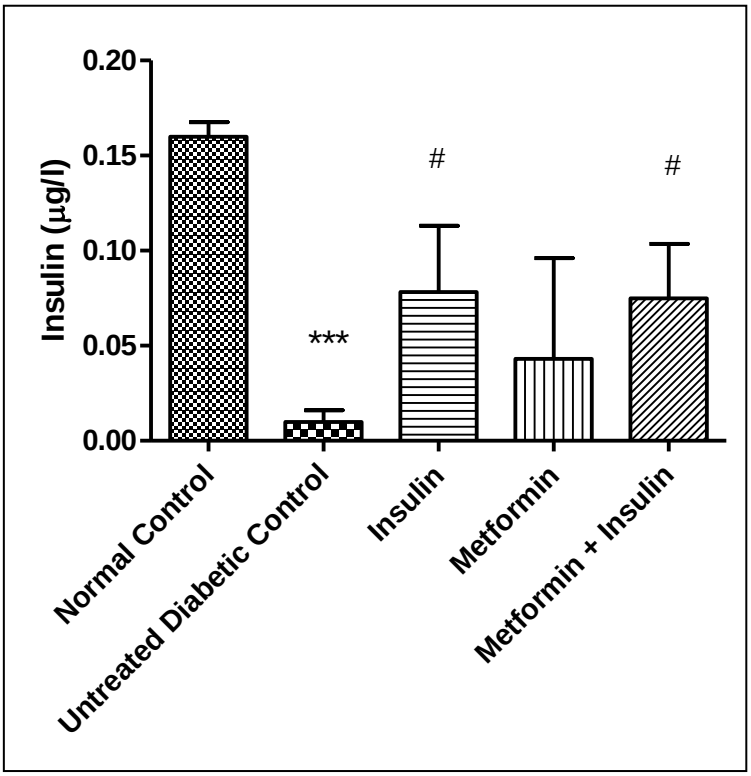

Figure 11: Fasting plasma insulin concentration. Plasma insulin concentrations were measured in blood obtained via cardiac puncture. ***$p < 0.0001$ compared to normal control ; # $p < 0.05$ compared to untreated diabetic control.

3.7 SERUM AND URINARY ELECTROLYTES

3.7.1 **URINARY ELECTROLYTES**

Urinary Na^+ output was significantly ($p<0.0001$) reduced in untreated diabetic animals compared to normal controls (Fig 9a). However treatment with insulin or metformin ($p < 0.05$) or metformin + insulin ($p < 0.0001$) significantly improved urinary Na^+ excretion compared to untreated diabetic controls (Fig 9a).

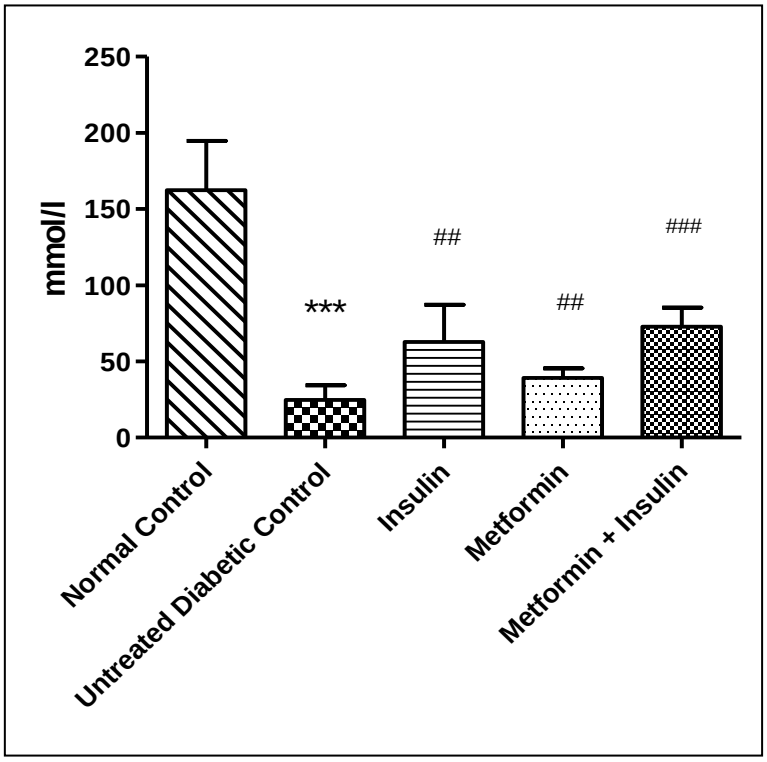

Figure 12a: Urinary Na^+ output. Animals were placed in metabolic cages for 24 hours and urine collected for analysis. Electrolyte concentrations were determined using diagnostic kits.

*** $p< 0.0001$ compared to normal control. $^{###}$ $p<0.0001$ compared to untreated diabetic controls. ## $p < 0.05$ compared to untreated diabetic controls.

Urinary K^+ output was significantly ($p< 0.0001$) reduced in untreated diabetic controls compared to normal controls (Fig 9b). However treatment with insulin or insulin + metformin significantly ($p < 0.05$) increased urinary K^+ concentration compared to untreated diabetic rats.

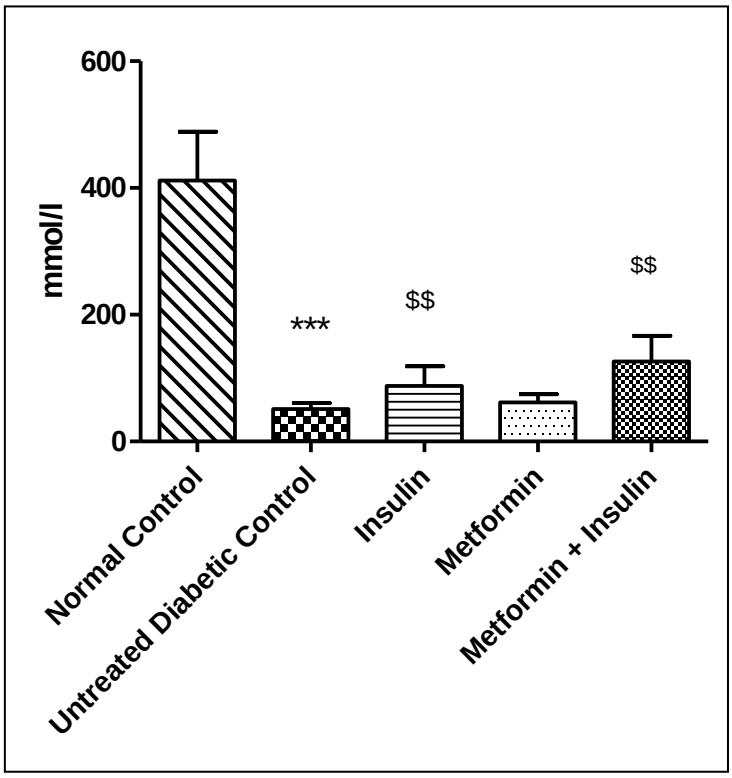

Figure 12b: Urinary K^+ output. Animals were placed in metabolic cages for 24 hours and urine collected for analysis. Electrolyte concentrations were determined using diagnostic kits.

*** $p< 0.0001$ compared to normal controls, $^{\$\$}$ $p < 0.05$ compared to untreated diabetic control

Urinary Cl^- output was significantly ($p < 0.0001$) reduced in diabetic controls compared to normal controls (Fig 9c). However, treatment with metformin + insulin significantly ($p < 0.001$) increased urinary Cl^- concentration, improving Cl^- excretion compared to untreated diabetic rats (Fig 9c).

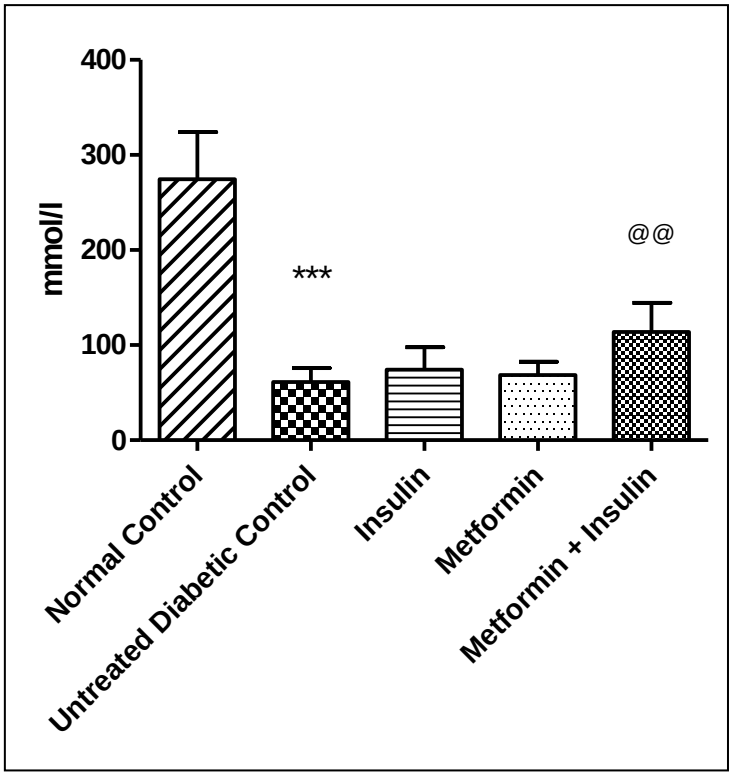

Figure 12c: Urinary Cl^- output. Animals were placed in metabolic cages for 24 hours and urine collected for analysis. Electrolyte concentrations were determined using diagnostic kits.

*** $p < 0.0001$ for diabetic control versus normal control. @@ $p < 0.01$ compared to untreated diabetic controls.

3.7.2 **SERUM ELECTROLYTES**

Diabetic controls exhibited significantly ($p < 0.0001$) reduced levels of serum Na^+ compared to normal controls. Treatment with metformin + insulin slightly but significantly ($p < 0.05$) increased serum Na^+ levels compared to the untreated diabetic animals.

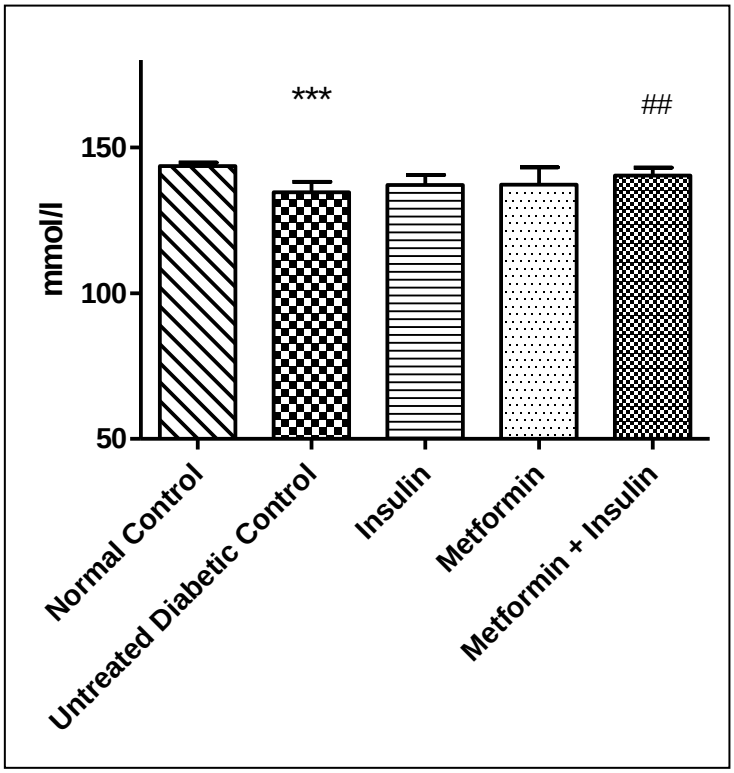

Figure 13a: Serum Na^+ concentrations. Serum electrolyte concentrations were determined using diagnostic kits. *** $p < 0.0001$ for untreated diabetic control versus normal control. ##$p < 0.05$ for metformin + insulin versus untreated diabetic control.

Serum K^+ levels were significantly elevated (p =0.01) in untreated diabetic controls compared to normal controls. Treatment with insulin with or without metformin significantly ($p<0.05$) reduced serum K^+ compared to untreated diabetic animals.

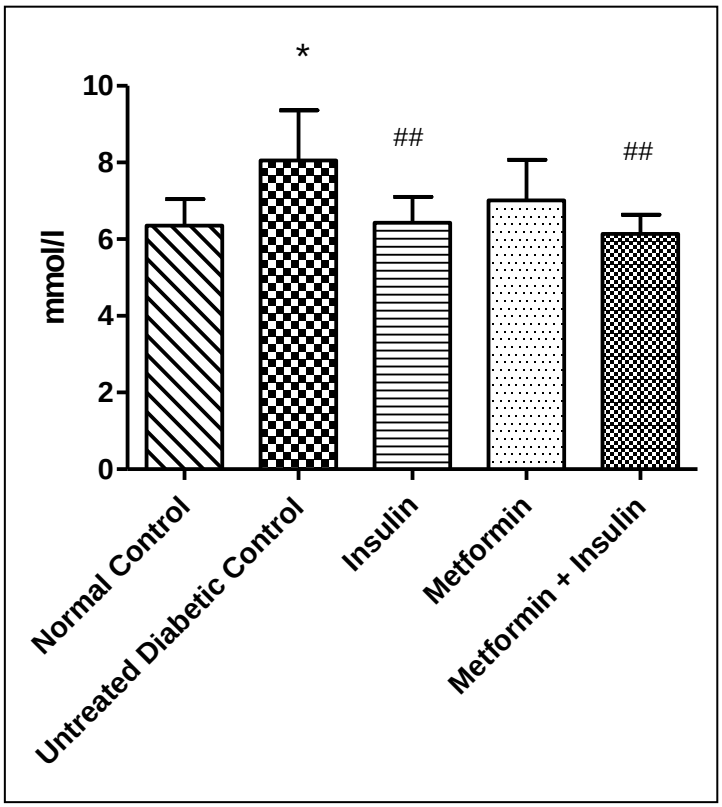

Figure 13b: Serum K^+ concentrations. Serum electrolyte concentrations were determined using diagnostic kits. * p= 0.01 compared to normal control. ## $p < 0.05$ compared to untreated diabetic controls respectively

Untreated diabetic controls exhibited significantly ($p<0.0001$) reduced serum Cl^- levels when compared to normal controls. Treatment with insulin or metformin did not improve serum Cl^- concentration in diabetic rats.

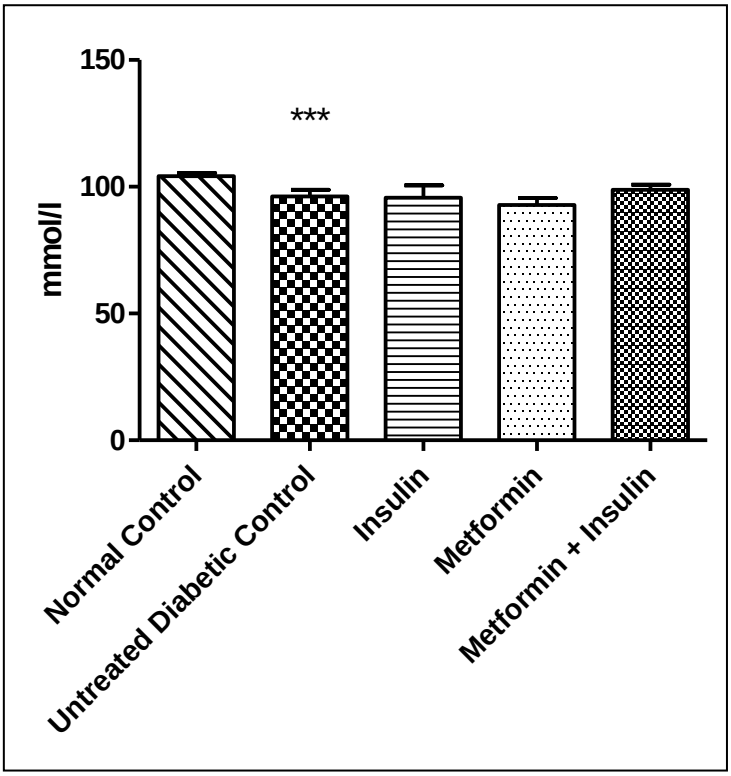

Figure 13c: Serum Cl^- concentration. Serum electrolyte concentrations were determined using diagnostic kits. *** $p < 0.0001$ compared to normal control.

3.8 SERUM AND URINARY CREATININE

3.8.1 **URINARY CREATININE**

Urinary creatinine output was significantly ($p < 0.0001$) reduced in untreated diabetic animals compared to normal controls. However, treatment with insulin with or without metformin significantly ($p < 0.01$) increased urinary creatinine excretion in diabetic animals compared to untreated diabetic animals (Fig 11). However, treatment with metformin alone did not significantly increase creatinine output.

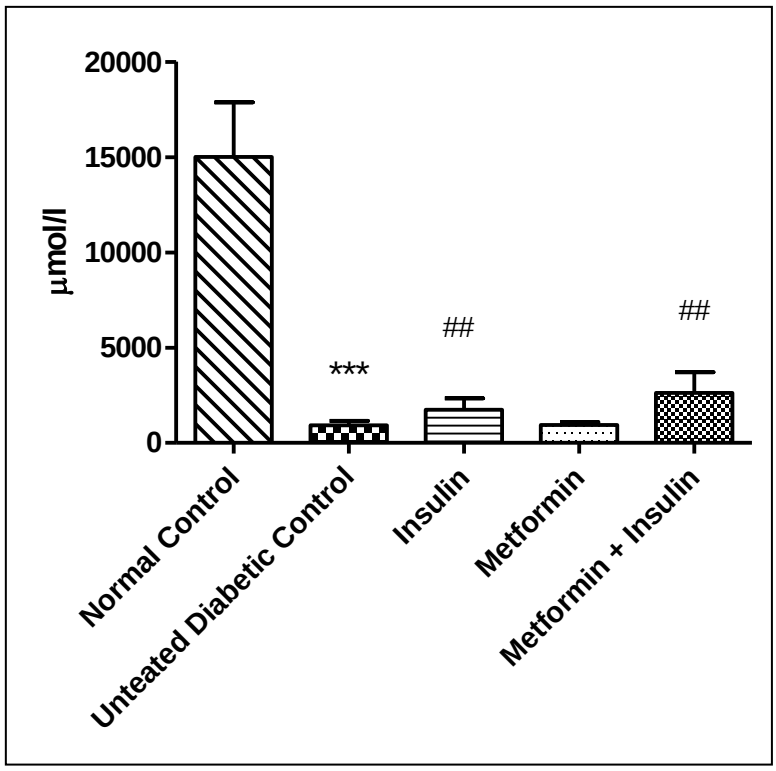

Figure 14: Urinary creatinine output. Creatinine levels were measured in urine samples which were collected over a period of 24 hours. *** $p< 0.0001$ for untreated diabetic controls versus normal controls. ## $p < 0.01$ compared to untreated diabetic controls respectively

3.8.2) **Serum Creatinine/ Weight**

As muscle mass decreases so does the concentration of creatinine as creatinine is a product of muscle metabolism. Serum creatinine was therefore corrected for weight differences across the groups; this was done by dividing the serum creatinine concentration by the final weight of each rat in each designated treatment group.

Serum creatinine to body weight ratio was significantly ($p<0.0001$) higher in the untreated diabetic rats compared to the normal controls (Fig. 15). However treatment with insulin with or without metformin significantly ($p<0.05$) reduced the serum creatinine to weight ratio compared to untreated diabetic animals (Fig. 15).

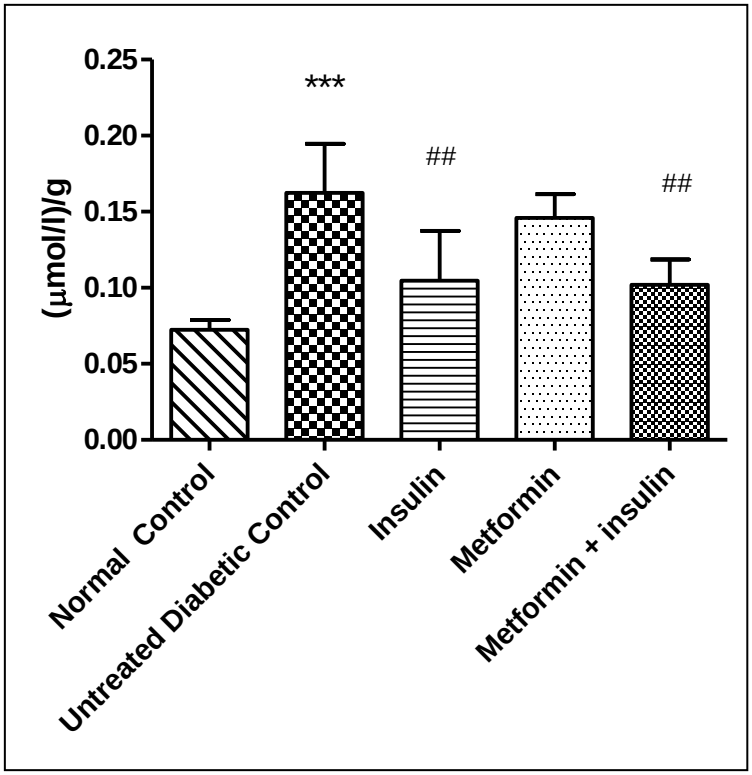

Figure 15: Serum Creatinine concentrations expressed as a ratio of creatinine to weight. Serum concentrations were divided by final weight of animal. *** $p<0.0001$ untreated diabetic control versus normal controls; ##$p < 0.005$ compared to untreated diabetic control

3.9 **UREA**

Untreated diabetic control rats had significantly ($p < 0.0001$) reduced urinary urea output compared to normal controls (Fig. 16). However, treatment with metformin + insulin significantly ($p<0.005$) raised urinary urea output compared to the untreated diabetic controls (Fig. 16). Treatment with insulin or metformin alone did not significantly increase urea concentrations compared to untreated diabetic animals.

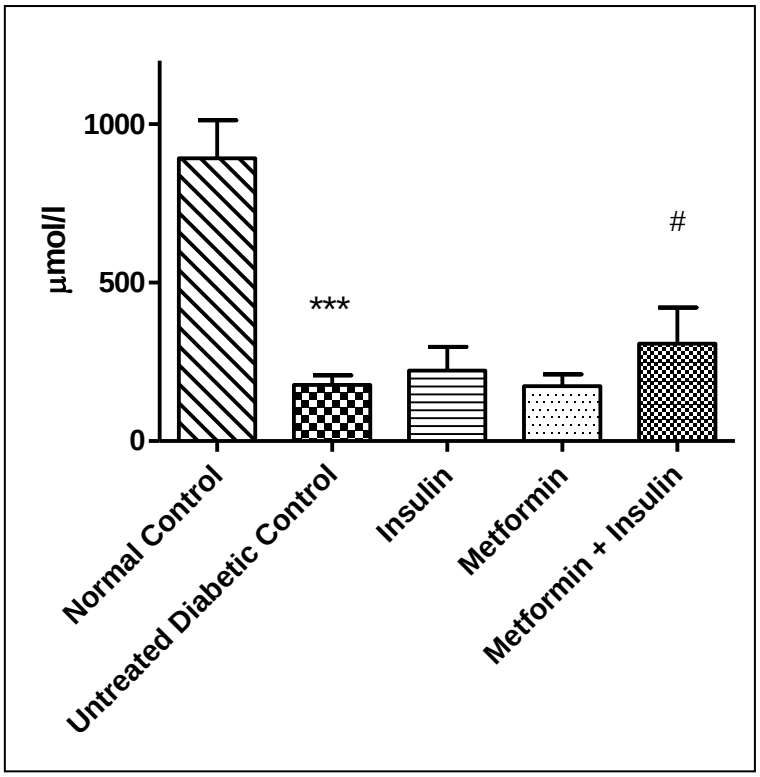

Figure 16: Urinary urea output. Animals were placed in metabolic cages for 24 hours and the urine collected. Urinary urea content was measured using diagnostic kits. *** $p< 0.0001$ compared to normal controls. # $p= 0.0121$ compared to untreated diabetic controls

3.10 **Calculated Creatinine Clearance**

Untreated diabetic controls had significantly ($p<0.05$) reduced creatinine clearance. However treatment with metformin + insulin significantly ($p< 0.01$) increased creatinine clearance (Fig. 17).

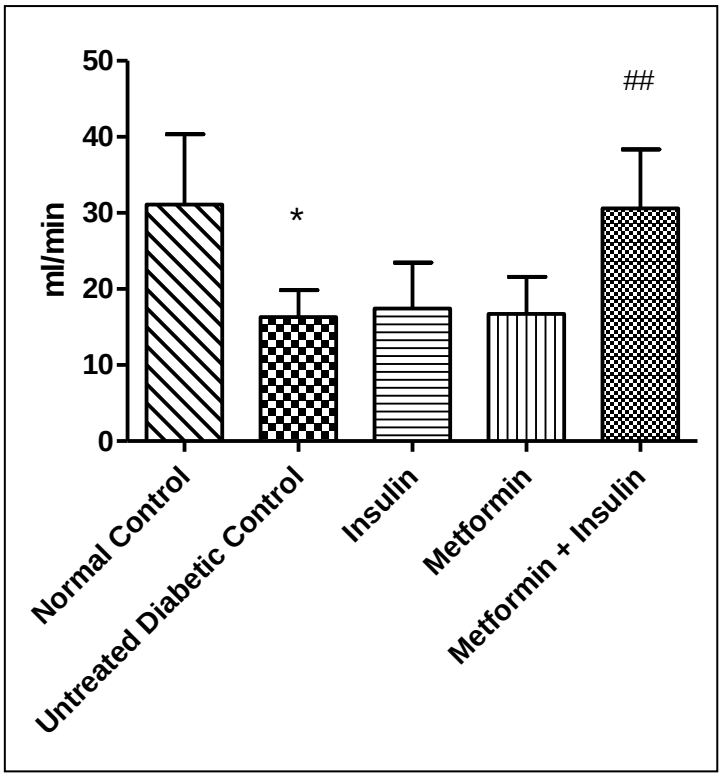

Figure 17: Calculated creatinine clearance. Creatinine clearance was calculated using the formula: (Cr conc urine/ Cr conc serum) x (urine volume/ time). *$p<0.05$ compared to normal control. ## $p < 0.01$ compared to untreated diabetic control

ASSESSMENT OF OXIDATIVE STRESS

3.11 **SUPEROXIDE DISMUTASE**

MnSOD activity was significantly increased in untreated diabetic controls ($p < 0.005$) compared to normal controls (Fig 15a). Treatment with metformin, with or without insulin, significantly ($p<0.001$) reduced MnSOD activity. Treatment with insulin alone however, did not significantly reduce MnSOD activity.

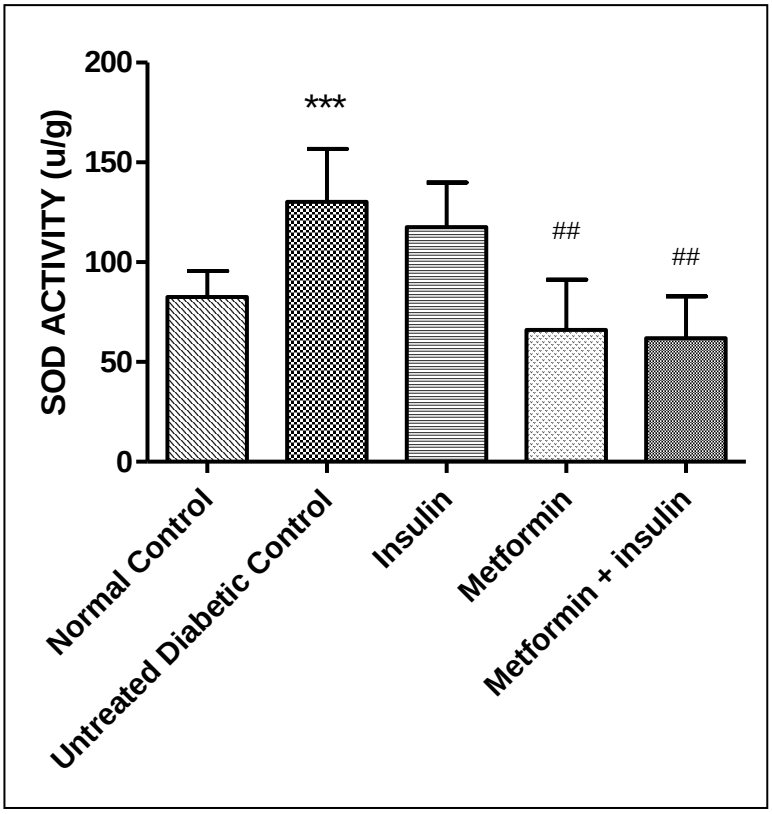

Figure 18a: Manganese Superoxide dismutase activity in renal tissue. Tissue was homogenised and measured as previously explained. *** $p < 0.005$ compared to normal control. ## $p< 0.001$ compared to untreated diabetic control.

Cu/ZnSOD activity was not significantly different between untreated diabetic and normal controls. Treatment with metformin + insulin resulted in a slightly reduced and significant ($p<0.05$) SOD activity compared to untreated diabetic animals.

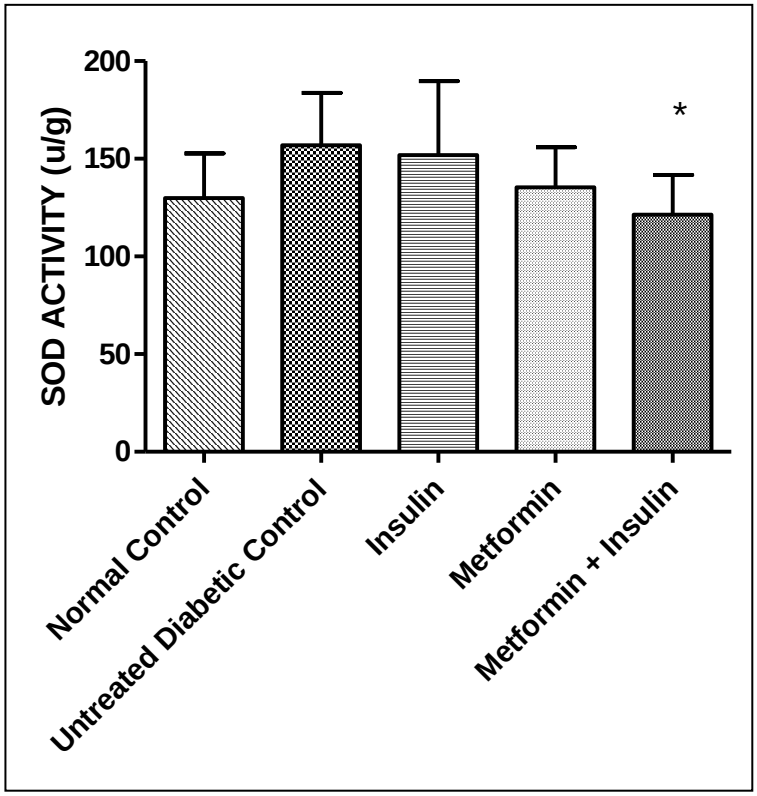

Figure 18b: Assessment of Copper/Zinc superoxide dismutase activity in renal tissue. Tissue was homogenised and measured as previously explained. *$p< 0.05$ compared to untreated diabetic control

3.12 **PLASMA LIPID PEROXIDATION**

Untreated diabetic controls experienced significant ($p<0.0001$), elevation of plasma levels of malondialdehyde compared to normal controls (Fig. 19a). Treatment with insulin, metformin or metformin + insulin significantly ($p<0.0001$) reduced plasma malondialdehyde concentrations compared to diabetic controls (Fig. 19 a).

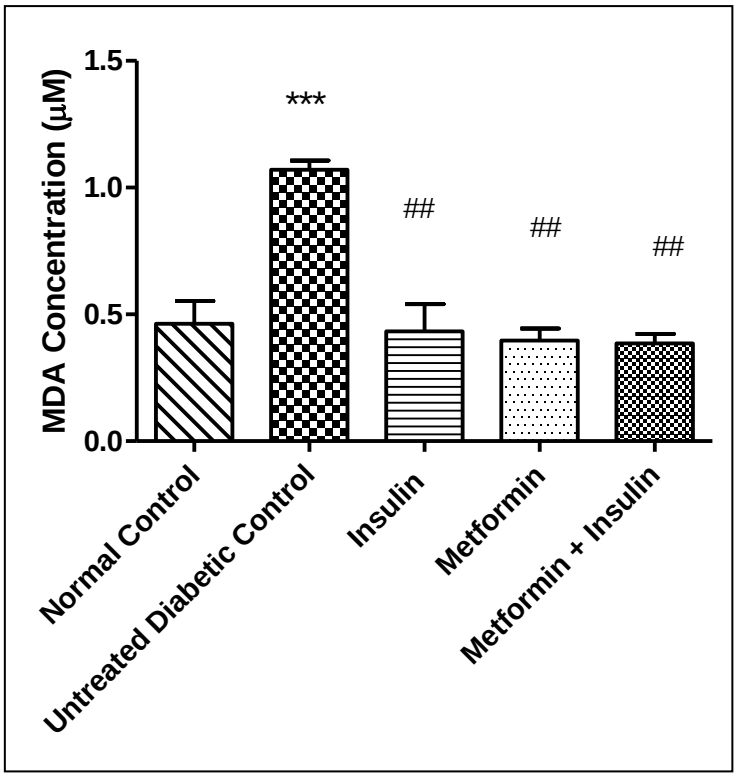

Figure 19a: Plasma concentrations of Malondialdehyde. Plasma samples were assayed and malondialdehyde concentrations determined. ***$p< 0.0001$ compared to normal control, ##$p<0.0001$ compared to untreated diabetic control.

3.13 **RENAL TISSUE LIPID PEROXIDATION**

Untreated diabetic controls exhibited significantly (p= 0.001) increased levels of renal tissue malondialdehyde concentrations compared to normal controls (Fig. 19 b). However, treatment with metformin, with or without insulin, resulted in significantly (p<0.0001) reduced levels of renal tissue malondialdehyde compared to the untreated diabetic rats.

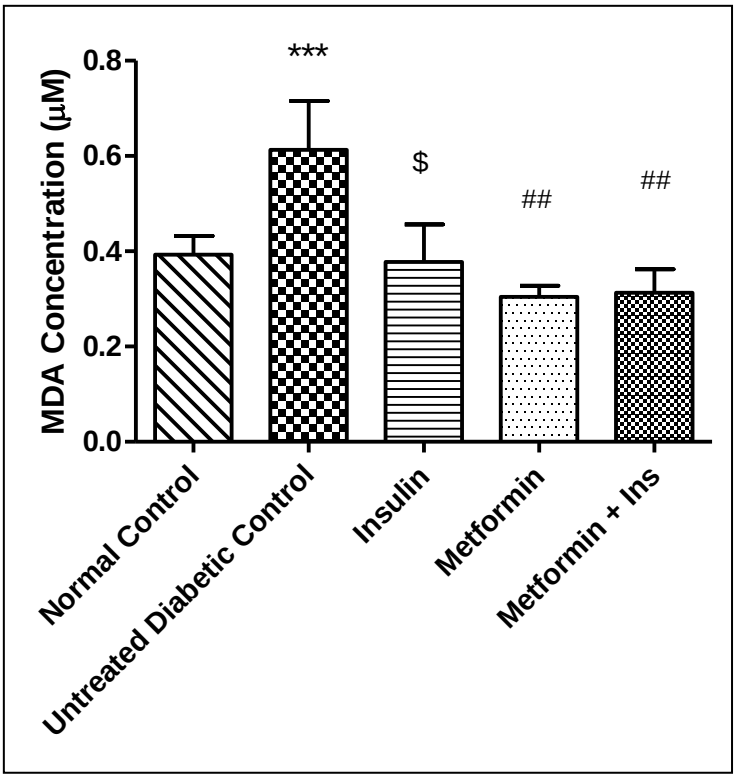

Fig 19b: Renal tissue concentrations of malondialdehyde. Renal tissue was homogenised and then assayed for lipid peroxidation concentration. *** p = 0.001 compared to normal controls. ## p< 0.0001compared to untreated diabetic controls. $ p< 0.05 compared to untreated diabetic controls

3.14 **RENAL TISSUE GLUTATHIONE CONCENTRATIONS**

Glutathione concentrations were significantly ($p<0.05$) reduced in untreated diabetic controls compared to normal controls. However, treatment with metformin ($p<0.05$) or metformin + insulin ($p<0.0001$) significantly increased glutathione concentrations in renal tissue.

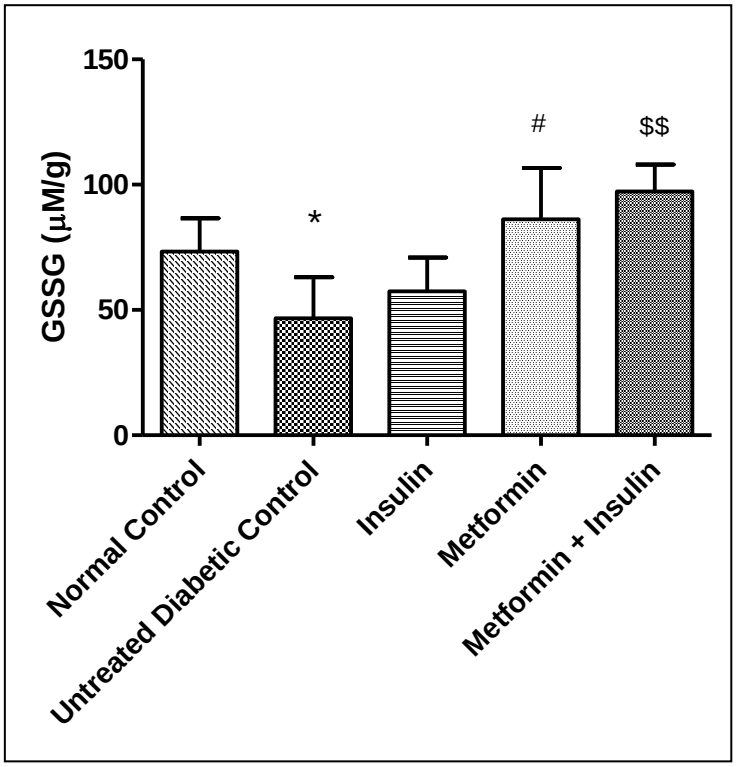

Figure 20: Renal tissue glutathione concentrations. Tissue was homogenised and glutathione concentrations measured as previously explained. *$p<0.05$ compared to normal control. #$p< 0.05$ compared to untreated diabetic control. $$ $p<0.0001$ compared to untreated diabetic control

CHAPTER FOUR:

DISCUSSION AND CONCLUSION

In this study we aimed to investigate whether metformin ameliorates aspects of diabetic nephropathy and improves renal function when added to insulin in the treatment of T1D. The results obtained indicate that metformin does have positive effects on renal function; there were increased concentrations of urine electrolytes with a concomitant decrease in serum electrolytes. There was improved creatinine clearance and an improved oxidative stress state. This was evident via a reduction in SOD activity and a reduction in malondialdehyde concentration when rats were treated with adjunctive metformin. However, metformin does not improve glucose intolerance in a type 1 diabetic model. Metformin improved oxidative stress associated with diabetes and prevented renal function deterioration. This adds further evidence to existing studies which claim that metformin possesses renopreotective properties [29, 68].

4.1 **Glucose Intolerance**

Type 1 diabetes was induced in the treatment groups via an intraperitoneal injection of streptozotocin at a dose of 65 mg/kg. At this dose streptozotocin, a cytotoxic glucose analogue completely destroys the pancreatic beta-cells [78]. These cells are responsible for insulin secretion under normal conditions. Streptozotocin is taken up selectively into the beta-cells through the GLUT-2 transporter and accumulates in the beta cells. Once in the cell, streptozotocin splits into glucose and methylnitrosurea [78]. The metylnitrosurea moiety possesses DNA alkylating activity. This causes DNA damage and ultimately leads to β-cell death [78]. At a dose of 65 mg/kg, streptozotocin causes complete beta-cell destruction [79].

Fasting plasma insulin levels were highest in the normal controls and lowest in the untreated diabetic controls (Fig.11). This was expected as there was total beta-cell destruction after streptozotocin administration in the diabetic groups. The groups treated with insulin, with or without metformin, had increased concentrations of plasma insulin compared to the diabetic control but lower than the normal control (Fig. 11). A reason for this is that animals were fasted overnight before being euthanized and did not receive treatment the morning of the euthanasia. Therefore plasma insulin levels reflected residual long-acting insulin still present in the plasma from the prior dose.

Classic T1D signs were displayed by all treatment groups that were made diabetic. These signs included weight loss (Fig. 6), polyurea (Fig. 8), polydipsia (Fig. 7) and high glucose levels (Fig. 9). This was expected because insulin is an anabolic hormone which promotes tissue growth. Therefore, in its absence the body goes into a catabolic state and rapid weight loss ensues [80]. As glucose cannot be taken up by the cells because of a lack of insulin, the body resorts to breaking down fat and protein for energy instead, this leads to muscle wasting [81]. Weight loss and muscle wasting are classic signs of type 1 diabetes. This accounts for the weight loss seen in the diabetic controls and the diabetics treated with metformin (Fig. 5 and Fig. 6). When insulin was present as in the normal controls, the insulin group and the group treated with metformin + insulin, animals' weight increased (Fig. 6). This is because insulin is a powerful anabolic hormone and causes weight gain. However, the diabetic control group and the group treated with metformin alone experienced overall weight loss due to a lack of insulin presence. Groups which were treated with insulin alone or a combination of insulin & metformin picked up weight steadily throughout the study, which was significant when compared to the untreated diabetic control (Fig. 6). Natural growth dipped and weight fell on days where rats were fasted overnight in preparation for Fasting Blood Glucose (FBG)

tests to be carried out (Fig. 5). As soon as normal diet was resumed normal growth patterns continued.

All diabetic groups in the study displayed classic polydipsia and polyuria. The hyperglycaemia present in T1D causes increased glucose concentrations in the renal tubules of the kidney, this leads to osmotic diuresis and polyuria which in turn stimulates polydipsia [80]. Polydipisia arises due to intracellular dehydration caused by elevated glucose levels [80, 81]. Both polyuria and polydipsia are classic symptoms of type 1 diabetes. Groups treated with insulin alone or a combination of metformin + insulin had lower levels of urine output compared to the diabetic control group (Fig. 8). This indicates that there were reduced levels of glucose present and hence, less osmotic diuresis and reduced urine output. Metformin, a drug commonly used in type 2 diabetes to reduce blood glucose levels had no effect when used alone on polyuria and polydipsia in this T1D model. This too could have been expected. Metformin has no diuretic properties and is an insulin sensitizer which requires the presence of insulin in order to lower glucose levels. Therefore, when metformin was used alone, hyperglycaemia remained high and the classic T1D signs persisted.

Fasting blood glucose levels were measured every 14 days throughout of study (Fig 9). Diabetic controls displayed progressively higher blood glucose levels. The insulin group and the group treated with metformin + insulin displayed lower blood glucose levels compared to the diabetic control. Fasting blood glucose levels for insulin group and the metformin + insulin group were relatively high compared to the normal controls. A suggested reason for this is that insulin treatment was not given prior to testing as it was intended that long-term residual effects of treatment be investigated and not short-term immediate effects. Hence, no treatment was given once fasting had commenced and up until after FBG tests were carried out.

When glucose tolerance tests were carried out the insulin treated and the metformin + insulin treated groups had a better response to the glucose dose compared the untreated diabetic control group. Between the two groups, the group treated with metformin + insulin started at a lower baseline glucose level and returned almost back to baseline faster. When AUC for the GTT was calculated, the metformin + insulin group had a significantly lower AUC compared to the untreated diabetic control (Fig. 10b). This indicates that the group treated with metformin + insulin was more tolerant towards a bolus glucose dose

In our study metformin enhanced the effects of insulin. Fasting blood glucose levels in the metformin + insulin group were lower than those in the group treated with insulin alone (Fig. 9); however, this difference was not statistically significantly. Treatment with metformin alone showed no improvement in glucose control. This was expected as metformin requires insulin in order to carry out its glucose lowering effects.

In other studies conducted where metformin was added as an adjunctive therapy to insulin in T1D, similar results were obtained [49, 82]. Studies have shown that when metformin is added to insulin for T1D it is associated with a decrease in the insulin dose required to maintain normal glycaemic levels [49, 50, 58, 82]. This is due to metformin's insulin sensitising activity. However, despite lowering insulin requirements, there is no long term decrease in HbA1c levels associated with adjunctive therapy in T1D [49, 50, 58]. The reasoning for this is that when metformin was added to insulin therapy in human based studies, patients reduced their insulin dose in order to maintain usual HbA1c levels [49].

Metformin enhances the effects of insulin by upregulating the translocation of glucose transporters GLUT-4 and GLUT-2 to the hepatic plasma membrane. Consequently this causes an increase in glucose uptake which further leads to increased glycogen synthesis and decreased gluconeogenesis [48]. It is also thought that metformin directly inhibits

gluconeogenesis through its effects on the mitochondrial electron transport chain and AMPK [48, 51, 83]. Metformin, through its inhibition of the mitochondrial electron transport chain, causes an increase in the AMP:ATP ratio. This results in an increase in AMPK activity and therefore glucose uptake is promoted and gluconeogenesis in inhibited. An increase in AMP furthermore leads to the suppression of adenylate glucose which down regulates PKA, a downstream activator of gluconeogenesis gene expression [51]. This is illustrated in our results where the groups treated with insulin or a combination of metformin + insulin had an average lower fasting blood glucose levels and a lower AUC for the glucose tolerance tests.

4.2 **Renal Function**

The kidneys' most important function is to maintain fluid and electrolyte balance within the body [84]. Our results showed that electrolyte (sodium, potassium and chloride) levels in the urine were significantly higher in the normal controls compared to the untreated diabetic control group (Fig 12 a, b, c). As renal function deteriorates there is increased retention of electrolytes and less being excreted in the urine. Retention of electrolytes can cause an electrolyte imbalance and may affect health. This can lead to a retention of waste products (urea and creatinine) and electrolytes (Na^+, K^+) which can have serious consequences such as adverse cardiac outcomes [84]. Hyperkalemia and hypernatraemia are common problems in renal dysfunction. Hyperkalemia can lead to serious complications such as hypotension and ventricular dysrhythmias which impact on the cardiovascular system [84]. Potassium and sodium levels in the urine, were significantly higher in both the insulin group and the metformin + insulin group compared to diabetic control (Fig. 12a, 12b), this possibly indicates that renal function was better preserved in these groups.

Serum potassium concentrations were elevated compared to normal controls; this was expected due to the decrease in potassium electrolyte excretion in urine (Fig 13. b). However, sodium serum concentrations were decreased in diabetic controls compared to normal controls (Fig. 13a). A suggested explanation for this is that due to an increased serum concentration caused by renal impairment and decreased electrolyte excretion via the urine, an electrolyte shift may have occurred where electrolytes shifted out the serum and entered the intracellular space instead. An osmotic shift may have also occurred due to cellular dehydration.

As creatinine concentrations were corrected for weight variations between groups it was seen that diabetic groups had higher levels of serum creatinine compared to the normal control (Fig. 15). Both the metformin + insulin group and the insulin treated group produced significantly lower levels of serum creatinine compared to the untreated diabetic controls. An elevated serum creatinine indicates impaired kidney function due to inadequate clearance. As the kidneys are responsible for creatinine clearance, as renal function deteriorates serum creatinine levels begin to rise. Investigating serum creatinine concentrations provides an indication of kidney function. Creatinine is a product of muscle metabolism and under normal circumstances is filtered at the glomerulus and also in small amounts in the proximal tubule [85, 86]. If renal function is compromised, creatinine will not be fully excreted and may accumulate in the body. This explains the results we obtained. In the insulin treated and the metformin + insulin treated groups, kidney function experienced less deterioration and more resembled the normal control. They had significantly less serum creatinine compared to the untreated diabetic control (Fig. 15). Creatinine levels would also rise in the presence of increased muscle catabolism. This is common in T1D where in the absence of insulin the body is unable to utilise glucose for energy and resorts to fat and muscle breakdown instead.

Creatinine clearance is a good estimator of glomerular filtration rate. Creatinine clearance measures the volume of plasma cleared of creatinine by the proximal tubule as well as excreted by the glomerulus per minute [85]. As it is not possible to measure glomerular filtration rate (GFR) directly, creatinine clearance is used to estimate GFR. GFR measures the rate at which substances are filtered out of the blood from the glumeruli and into the Bowman's capsule of the nephrons [85]. GFR provides a good indicator of renal function with a reduction in GFR indicating functional impairment of the nephrons [86-88]. As renal function decreases, creatinine clearance as well as GFR also decreases. In this study creatinine clearance was highest in the normal controls and the group treated with metformin + insulin (Fig. 17). The untreated diabetic control group produced the lowest creatinine clearance rate. When metformin was combined with insulin, creatinine clearance significantly improved. This indicates that treatment with metformin + insulin helped preserve renal function and caused an improvement in creatinine clearance.

Urea is a water-soluble molecule which is an end product of protein metabolism [87]. It is found highly concentrated in the urine. If kidney function is compromised one would expect a decrease in the concentrations of urea found in the urine. Urea concentrations were significantly lower in the untreated diabetic control group compared to the normal control group. The group treated with metformin + insulin displayed significantly increased concentrations of urea compared to the untreated diabetic control (Fig. 16). It is known that gluconeogenesis in the kidney is also closely linked to ureagenesis. This is to compensate for the increase in nitrogen and amino acids created in gluconeogenesis [87]. It is also thought that glucagon, the hormone which promotes gluconeogenesis also stimulates urea synthesis by the liver [87]. Therefore one would initially expect the urea concentrations to be elevated in the diabetic control group and in groups where glucose control was not fully obtained. However, in our results urea concentrations were elevated in the normal control group and

lower in the untreated diabetic control group. There are two possible explanations of this. Firstly, the diabetic groups produced more urine compared to the untreated control group suggesting the presence of polyuria caused by hyperglycemia. Although there possibly could have been more urea in the diabetic groups, concentration remained low due to an increased urine volume (a dilutionary effect). Secondly, renal function has probably deteriorated in the diabetic groups and the kidney's ability to filter out this by-product of protein metabolism may have been compromised, resulting in decreased urea concentration in the urine. Added to this is the reduced body mass found in the diabetic control group and the group treated with metformin. As urea is an end-product of protein metabolism a reduced body mass would also lead to reduced levels of urea being produced and excreted and could have also contributed to the finding. Unfortunately serum urea was not investigated in this study; however, one would expect serum urea to be elevated due to decreased urea excretion in urine.

Previous studies have shown metformin to possess renoprotective effects. Metformin has been shown to attenuate gentamycin induced nephropathy, as well as protect against podocyte and renal tubular injury [29, 31, 68, 69]. It is thought to achieve this by reducing oxidative stress via activation of AMPK and inhibiting complex 1 of the mitochondrial electron transport chain [68, 69]. Some *in vitro* studies have shown that when metformin is incubated along with an AMPK inhibitor, metformin's antioxidant effects were completely abolished [31]. Piwkowska et al. (2010) demonstrated that podocytes incubated with metformin inhibited the formation of superoxide to a similar degree to when podocytes were incubated with an NADPH- oxidase inhibitor and further showed that when compound C (an AMPK inihibitor) was administered, metformin had no effect on NADPH oxidase activity [31]. Hence there could be a link between AMPK activation and NADPH-oxidase superoxide production [31].

As to the best of my knowledge, no studies have examined the effects of metformin on electrolyte concentrations, creatinine clearance or urea concentration before in relation to the development of renal impairment in T1D.

4.3 **Oxidative Stress**

Hyperglycemia has been shown to decrease the body's total antioxidant capacity and increase oxidative stress [20]. This increase in oxidative stress together with prolonged hyperglycemia increases the prevalence of diabetic complications [16, 21]. SOD has been shown to be a powerful antioxidant and therefore an indicator of oxidative stress [3, 18]. Manganese superoxide dismutase is found in the mitochondria whereas copper/zinc superoxide dismutase is commonly found in the cytoplasm. Superoxide dismutase activity was measured in order to investigate the state of oxidative stress present.

In our study the normal controls displayed significantly less SOD activity, with the untreated diabetic control group displaying the most activity (Fig 18 a, b). The group treated with metformin + insulin as well as the group treated solely with metformin had significantly less Mn-SOD activity compared to the untreated diabetic control (Fig. 18 a). Groups treated with metformin, with or without insulin, had even lower SOD activity than the normal control. This is because metformin acts predominantly in the mitochondria [52, 70]. Metformin acted to decrease the oxidative stress via its action on AMPK and the ETC, therefore SOD activity was reduced. Metformin inhibits complex 1 of the mitochondrial ETC which leads to inhibition of mitochondrial ROS production [45]. As there was more oxidative stress present in the diabetic control, there was an increase in SOD activity in order to combat the oxidative stress. In the normal control there was reduced oxidative stress therefore SOD activity

remained low. Superoxide dismutase is an inducible enzyme and therefore; in non oxidative stress conditions will not be activated.

Copper/zinc SOD is predominantly found in the cytosol. Superoxide dismutase activity was slightly decreased in the metformin + insulin group as well as the normal control group; however, the difference was of little significance (Fig 18 b). This is likely explained by the fact that metformin does not act in the cytosol but rather in the mitochondria. Therefore, Cu/Zn-SOD activity was not reduced to any great extent as activity was limited to the mitochondria and not the cytosol.

Malondialdehyde (MDA) is a frequently used indicator of lipid peroxidation and is a biomarker for oxidative stress. Plasma malondialdehyde concentration is a good indicator of the presence of total oxidative stress. The present study showed that MDA plasma concentrations were highest in the diabetic control group and lowest in the group treated with metformin + insulin (Fig. 19 a). All groups had significantly lower MDA concentrations when compared to the untreated diabetic control group. This further suggests that metformin operates as an antioxidant to reduce oxidative stress. Treatment with metformin, with or without insulin greatly reduced MDA concentrations. The same pattern emerged when MDA concentrations were measured in renal tissue (Fig. 19 b). MDA concentrations were highest in the untreated diabetic control group. The normal controls as well as the groups treated with metformin and a combination of metformin + insulin had significantly reduced levels of MDA compared to the diabetic controls. These results further strengthen the thought that metformin reduced oxidative stress through antioxidant properties.

Glutathione is an important antioxidant and scavenger of ROS [19, 21]. GSH levels were elevated in the groups treated with metformin and lowest in the untreated diabetic control group (Fig. 20). Metformin + insulin treatment resulted in highly significant increases in GSH levels compared to the diabetic control. Despite GSH being a highly inducible enzyme, oxidative stress was highest in the diabetic control group which resulted in GSH being low. Metformin, potentially acting as an antioxidant, protected against oxidative stress; and thereby preserved GSH levels. Under hyperglycaemic conditions where oxidative stress is high, GSH levels are depleted. NADPH is used by aldose reductase in the polyol pathway of oxidative stress to form sorbitol. Glutathione reductase is therefore unable to use NADPH in order to reduce GSSG to GSH, leading to a depletion in the GSH pool [18, 22]. Therefore results in the present study indicate that oxidative stress was highest in the untreated diabetic control group and significantly reduced in the groups treated with metformin.

4.4 CONCLUSIONS

In this study we tested the ability of metformin to reduce or ameliorate diabetes associated renal impairment when used as an adjunct to insulin therapy in type 1 diabetes. The data presented here indicates that metformin does not improve glycaemic control in type 1 diabetes but does have protective effect against the development of renal impairment and diabetic nephropathy. There was an improvement in creatinine clearance, and there were increased concentrations of urine electrolytes with concomitant decreases in serum electrolytes. Also, when used in conjunction to insulin, metformin reduces oxidative stress and helps preserve renal function in diabetic rats. Treatment with metformin in conjunction with insulin reduced SOD activity, reduced MDA concentration in plasma and renal tissue as well as increased glutathione concentration in renal tissue.

4.5 STUDY LIMITATIONS

Short comings of the study include a lack of histological tests and diabetic nephropathy markers such as albumin levels. However, this study does prove a need to further advocate the use of metformin in T1D for the alleviation of diabetic complications such as diabetic nephropathy and renal impairment. Further studies are warranted to investigate fully the use of metformin in T1D to prevent or delay the onset of diabetic nephropathy and the other complications that results from T1D.

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APPENDICES

Appendix 1: Ethics approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

31 March 2014

Reference: 078/14/Animal

Dr P Owira
Dept of Pharmacology
School of Health Sciences
Room 521 – Block F2
WESTVILLE Campus

Dear Dr Owira

RENEWAL: Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Research Ethics Committee has granted ethical approval for **2014** on the following project:

"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 Research Ethics Committee

Cc Registrar – Mr C Baloyi
Research Office – Dr N Singh
Head of School – Prof. S Essack
BRU – Dr S Singh

Animal Ethics Committee
Professor Theresa HT Coetzer (Chair)
Postal Address: Room 105, John Bews Building, Private Bag X01, Pietermaritzburg, 3201, South Africa
elephone: +27 (0)33 260 5463/35 Facsimile: +27 (0)33 260 5105 Email: animalethics@ukzn.ac.za Website: www.ukzn.ac.za
Edgewood Howard College Medical School Pietermaritzburg Westville

Appendix 2: Abstract College of Health Sciences Research Symposium, 10-11 September 2015

METFORMIN DOES NOT IMPROVE GLUCOSE INTOLERANCE BUT AMELIORATES ASPECTS OF DIABETIC NEPHROPATHY BY REDUCING OXIDATIVE STRESS IN TYPE 1 DIABETES.

Driver C and Owira PMO

Department of Pharmacology, Discipline of Pharmaceutical Sciences, School of Health Sciences

Introduction

Type 1 diabetes (T1D) is a chronic condition caused by the complete destruction of insulin producing pancreatic β-cells. Increased oxidative stress and impaired antioxidant capacity are associated with diabetic complications. Metformin, commonly used to treat type 2 diabetes, has been suggested to have antioxidant capacity. We hypothesize that metformin as an adjunct to insulin in T1D may help prevent the development of diabetic complications such as nephropathy by decreasing oxidative stress.

Aim

To investigate metformin's effect on oxidative stress and diabetic nephropathy when used as an adjunct to insulin in T1D.

Method

Sprague-Dawley rats were divided into 5 groups (n=7), (A: Vehicle control, B: Diabetic control, C: T1D+Insulin (4U/kg bd), D: T1D+Metformin (250mg/kg via oral gavage), E:T1D+Metformin+Insulin). Diabetes was induced by intraperitoneal streptozotocin injection (65 mg/kg body weight) and confirmed after 48 hrs later. Glucose Tolerance Test (GTT), serum and urinary electrolytes (K^+, Cl^-, Na^+), creatinine, urea and superoxide dismutase activity were analysed.

Results

Metformin alone did not improve glucose intolerance but with insulin significantly showed increased electrolyte excretion and also greatly improved creatinine clearance when compared to the diabetic control group. Metformin with insulin further reduced superoxide dismutase activity compared to diabetic control.

Conclusion

Metformin has positive additive effects on oxidative stress and diabetic nephropathy when used as an adjunct therapy to insulin for T1D treatment.

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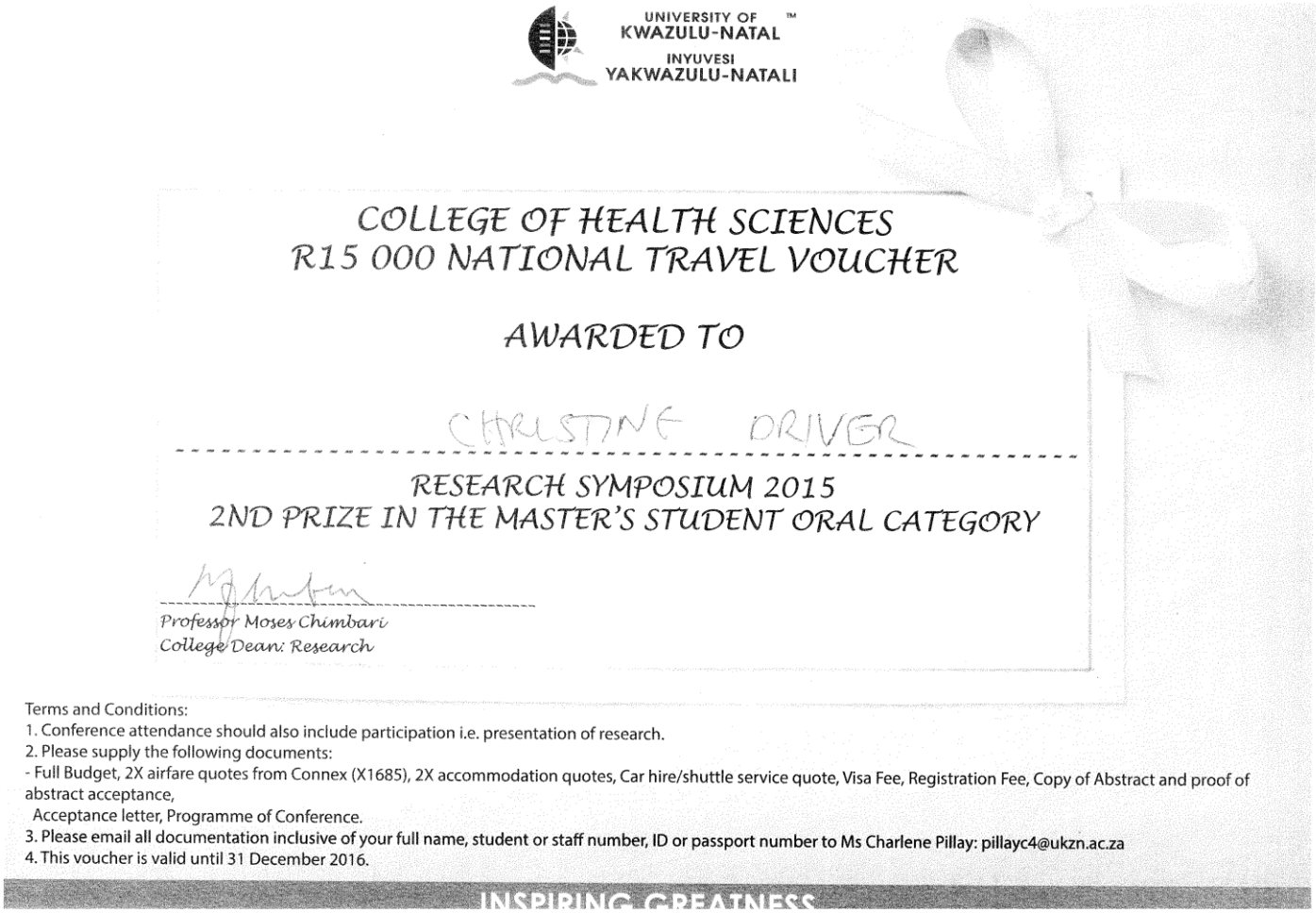
Appendix 3: College of Health Sciences Research Symposium Award Certificate (2nd prize master's student oral category)



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Acceptance letter, Programme of Conference.
3. Please email all documentation inclusive of your full name, student or staff number, ID or passport number to Ms Charlene Pillay: pillayc4@ukzn.ac.za
4. This voucher is valid until 31 December 2016.

INSPIRING GREATNESS

Appendix 4: Manuscript Draft, "Naringin does not Improve Glucose Intolerance but Mitigates Oxidative Stress and ketoacidosis in type 1 Diabetic Rats"

PLOS ONE

GRAPEFRUIT-DERIVED FLAVONOID NARINGIN IMPROVES KETOACIDOSIS AND LIPID PEROXIDATION IN TYPE 1 DIABETES RAT MODEL

--Manuscript Draft--

Manuscript Number:	PONE-D-16-00381R1
Article Type:	Research Article
Full Title:	GRAPEFRUIT-DERIVED FLAVONOID NARINGIN IMPROVES KETOACIDOSIS AND LIPID PEROXIDATION IN TYPE 1 DIABETES RAT MODEL
Short Title:	Naringin ameliorates diabetic ketoacidosis
Corresponding Author:	Peter MO Owira University of Kwazulu-Natal Durban, KZN SOUTH AFRICA
Keywords:	Naringin, antioxidants, ketoacidosis, hyperglycemia.
Abstract:	Background Hypoglycemic effects of grapefruit juice are well known but the effects of naringin, its main flavonoid on glucose intolerance and metabolic complications in type 1 diabetes are not known. Objectives To investigate the effects of naringin on glucose intolerance, oxidative stress and ketonemia in type 1 diabetic rats. Methods Sprague-Dawley rats divided into 5 groups (n=7) were orally treated daily with 3.0 ml/kg body weight (BW)/day of distilled water (group 1) or 50 mg/kg BW of naringin (groups 2 and 4, respectively). Groups 3, 4 and 5 were given a single intra-peritoneal injection of 60 mg/kg BW of streptozotocin to induce diabetes. Group 3 was further treated with subcutaneous insulin (4.0 IU/kg BW) twice daily, respectively. Results Stretozotocin (STZ) only-treated groups exhibited hyperglycemia, polydipsia, polyuria, weight loss, glucose intolerance, low fasting plasma insulin and reduced hepatic glycogen content compared to the control group. Furthermore they had significantly elevated Malondialdehyde (MDA), acetoacetate, β-hydroxybutyrate, anion gap and significantly reduced blood pH and plasma bicarbonate compared to the control group. Naringin treatment significantly improved Fasting Plasma Insulin (FPI), hepatic glycogen content, malondialdehyde, β-hydroxybutyrate, acetoacetate, bicarbonate, blood pH and anion gap but not Fasting Blood Glucose (FBG) compared to the STZ only-treated group. Conclusions Naringin is not hypoglycemic but ameliorates ketoacidosis and oxidative stress. Naringin supplements could therefore mitigate complications of diabetic ketoacidosis.
Order of Authors:	Peter MO Owira
	Alfred N Murunga
	David O Miruka
	Christine N Driver
	Fezile S Nkomo
	Snazo ZZ Cobongela
Opposed Reviewers:	
Response to Reviewers:	5. Review Comments to the Author Please use the space provided to explain your answers to the questions above. You may also include additional comments for the author, including concerns about dual publication, research ethics, or publication ethics. (Please upload your review as an attachment if it exceeds 20,000 characters)

Reviewer #1: The present study reported Narigin may improve the oxidative stress and ketoacidosis but have no effect on glucose tolerance in type 1 diabetic rat, which is interesting and challenging. There are still some concerns need to be addressed.
1.In the current study, hypoglycemic effect of Naringin was not observed which was inconsistent with other reports. More discusses are needed to interpret the increased insulin and hepatic glycogen but no influence on glucose by Naringin treatment. Would the potential hypoglycemic effect of Naringin exert on mild condition? Mild diabetic rats are suggested to use as an additional group (e.g.STZ 35 mg/kg). Have the authors try to confirm its insulinotropic effect on cell lines? Or other pathways for example, stimulating the secretion of incretin when oral GTT performed? (J endocrinology, 2013,217:185-196)
Response:
As highlighted in our discussion, hypoglycemic effects of naringin so far demonstrated are apparent in type 2 diabetes rat models where there is some residual insulin secretion. We have previously shown that without insulin, naringin does not exert hypoglycemic effects {J Cardiovasc Pharmacol. 2012 Feb;59(2):133-41; Accepted Manuscript in PLOS ONE (PONE-D-15-28866R2)}. Enhanced insulin secretion in normal non-diabetic rats treated with insulin could be attributed to antioxidant potential of naringin that reduces reactive oxygen species concentrations in the pancreatic β-cells leading to increased ATP production and insulin secretion (our as yet unpublished work on the effects of naringin on insulin secretion in RIN-5F cell lines). Insulinotropic effects of naringin have not previously been fully investigated but is a possibility. DPP-IV inhibitory potential of naringin has recently been described (Diabetes Res Clin Pract. 2012 Jul;97(1):105-11) suggesting that naringin could have increased insulin secretion in these rats by increased GLP-1 half-life. However, enhanced insulin secretion was an unexpected finding that was beyond the scope of our study in the interim.
Similarly, we unexpectedly found that naringin significantly increased glycogen content in non-diabetic rats compared to controls. However, this is not surprising considering our recent finding (Eur J Nutr. 2016 Mar;55(2):631-638) that grapefruit juice significantly increases hepatic glycogen content in non-diabetic and diabetic compared to controls and diabetic rats, respectively by activating glucokinase and down-regulating hepatic activities of key gluconeogenic enzymes, PEPCK and G-6-Pase. Interestingly, insulin treatment of diabetic rats did not significantly increase glycogen concentrations compared to untreated diabetic rats. Naringin is the predominant bioflavonoid in the grapefruit juice and we are convinced that in the previous and present study, it activated glycogen synthase via AMPK upregulation like metformin (Arch Biochem Biophys. 2012 Feb 1;518(1):61-70). These points have been incorporated into the discussion in the present manuscript.
2.STZ treatment may take some days to exert effects stably (generally 5-7 days). Why the authors confirm the blood glucose on day 3 after STZ treatment? Similarly, treatments were performed immediately following the confirmation. STZ induced diabetic model may lead to wide variations in weight and FBG. Some rats may display very severe symptom (e.g. FBG > 18 mmol/L, weight <180 g) following the STZ treatment. How did authors deal with this? Details of grouping are needed.
Response:
We have been consulted by other research groups on how we manage to induce stable hyperglycemia within 24 hrs of administering STZ without animal deaths. In our experience, the animals have to be fasted overnight, and STZ dissolved in 0.2 ml of 0.1 M citrate buffer, pH 4.5. It is critical that be buffer be made a fresh and the pH kept at 4.5 without adjusting with either NaOH or NaCl. In this way blood glucose concentrations remain stable and no adverse effects have been experienced in our studies
3.For the weight, water intake and urine output changes showed, it is better to provide a weekly line chart which may reflect the details and fluctuations.
Response:
We have previously tried that but the figures become cluttered due to large number of animals and the critical information would be blurred. Fluctuations in weights, water consumptions and urine outputs do occur during treatment in response to such situations as overnight starvation but the overall terminal changes are more critical in conveying the intended message than piecemeal effects of interventions.
4.Please unify the markers used for indicating significance which were disordered and confusing (&@*#^). For instance, may use # for comparing with normal control; * for untreated diabetic group;
Response:

Done
5.Abbreviations should not be used when the item firstly appears. Line 39, FPI; Line 138, FBG.
Response:
Done
Reviewer #2: The authors report the effects of 40-day daily treatment with the main flavonoid of grapefruit, Naringin, to a cohort of SZT-injected rats as a model of Type-1 Diabetes. Control cohorts included SZT-treated and normal rats, given either water or naringing, as well as a reference cohort of SZT-treated rats given insulin twice daily. After the 40-day treatment the cohorts were subjected to IPGTT using 3.0 g/kg dextrose by intraperitoneal injection. The next day the animals were sacrificed and blood was collected for a number of measurements, including insulin, ions, and metabolites. The authors describe the differences in ketoacidosis biomarkers between the Naringing-treated cohorts and control rats, suggesting that this treatment may improve ketoacidosis, which is the main message of this manuscript. The molecular target and physiological mechanism is not understood, and this cannot be known from exploratory in vivo experiments. However, the authors go to great lengths to hypothesize, but this seems unnecessary and potentially the most deterring aspect of the lengthy discussion section. The authors report and underscore a lack of significant differences between Naringin-treated and control rats in the IPGTT experiment. However, because of the relatively large dose of sugar used (in particular for a T1D model) this single GTT experiment is insufficient to fully support the claim of the authors. One further recommendation is changing the manuscript title to represent only the positive observations, for example: “Grapefruit compound Naringin improves ketoacidosis metabolites and lipid oxidation and in Type-1 Diabetes rat model”.
Editorial recommendation: major revisions.
Response:
We have previously reported inability of naringin to improve glucose intolerance in rats treated with STZ after a similar glucose challenge (J Cardiovasc Pharmacol. 2012 Feb;59(2):133-41) hence our observation here is not isolated. Naringin has not be demonstrated to be an insulin secretagogue and its proposed insulinotropic effects are apparent only in simulated type 2 diabetes models (Diabetes Res Clin Pract. 2012 Jul;97(1):105-11). Currently available scientific evidence suggests that naringin and its aglycone naringenin could be acting like insulin sensitizers just like metformin (Arch Biochem Biophys. 2012 Feb 1;518(1):61-70; Biochem Biophys Res Commun. 2010 Jul 23;398(2):178-83) by activating AMPK. In this context, it is not surprising that we did not observe improved glucose tolerance in a type 1 diabetes rat model. It cannot be argued that 3.0 g/kg body weight glucose used in this study was too high to show differences in IPGTT since even in non-STZ-treated rats, there were no significant difference in glucose tolerance between naringin-treated and control rats. A lower dose of glucose could have produced a flat curve considering the difference in blood glucose concentrations between STZ-treated and non-STZ-treated rats.
With anticipated permission of the Editor, we have changed the title accordingly
Main comments:

1)The introduction and discussion are too long and particularly focused on ketoacidosis as the main source of all ailments in diabetes. Major revision is required to remove sensationalistic language, please use ONLY standard scientific language that describes facts and credit associated literature references. To be very specific, replace the words/sentences: “courtesy”, “exacerbate”, “catabolic nature”, “contentious issues”, “random blood glucose”, “diabetic state”; as well as convoluted/incorrect concepts like “pro-hyperglycemia insulin counter-regulatory hormones”, “freely producing hydrogen ions which bind an overwhelm serum bicarbonate buffering capacity eventually leading to metabolic acidosis”, “hyperglycemia-associated oxidative stress causes lipid peroxidation due to increased ROS leading to overproduction of MDA”. There are also a number of unsupported statements like “offer some hope”, “devoid of adverse effects”, “abrogated metabolic acidosis”, “metformin-like effects” and “demonstrated powerful antioxidant effect”. Note that “powerful” is not an objective and meaningful word in this context (i.e. low redox potential of the molecule? binds with high affinity to a target?).
Response:
Noted. The offensive words/phrases have been purged where appropriate and the introduction and discussion sections shortened. However, we need to point out that the study was designed primarily to investigate the effects of naringin on diabetic

	ketoacidosis with glucose intolerance as a confirmation of diabetes and oxidative stress as a possible mechanism of action. Diabetic ketoacidosis is not a source of diabetes but rather a metabolic complication besides nephropathy, cardiomyopathy, retinopathy and neuropathy which we were not intent on investigating in this setting. We see no problem with using some of the phrases such as “random blood glucose” which is clinically applicable as opposed to “fasting blood glucose”. Similarly we see nothing wrong with referring to naringin as having ‘metformin-like effects” in the control of blood glucose as it appears to be an emerging consensus among different investigators in this field. 2)All instances of the sentence “Diabetic groups” (or “diabetic rats”) should be changed to “SZT-treated group”. Similarly, “untreated diabetic group” would be substituted with “SZT-treated control cohort”, etc. Response: Done 3)Figure 1 shows the BW at the end of the study, please show a longitudinal scatterplot. Figure 4 shows only the fasting glucose on day 28 of treatment, however, day 0 and 14 were also measured. This data should be included. Response: Done. 4) Fasting plasma insulin (FPI) was measured on day 42, which is 1-day post GTT on day 41. Therefore, this protocol must have required 2 nights of fasting in a row with a short ad libitium feeding time in between, which is not a standard recovery post-GTT. This needs to be clarified and stated because it may possibly lead to differences with other study designs in the future. Response: Fasting blood glucose were done on days 0, 14 28 and 41 but not 42 as part of IPGTT. The error has been corrected. The animals were then sacrificed on day 42. 4)The formulas for concentrations of [AcAc] and [3HB] are not consistent with concentration units. Please provide literature references for these methods. Please provide a reference for the quoted extinction coefficient of 1.56x10^5 for the MDA assay. Similarly, it would seem that the measurement of glycogen would require a standard curve to convert the spectrophotometer measurements to mg/g liver protein. Please include all data, literature references, and methods needed. Response: Anion Gap (AG) was calculated according to: Clin J Am Soc Nephrol. 2007 Jan;2(1):162-74, (reference no 11 in the revised manuscript) and expressed as mM (mmol/L) which is also the same as mEq/L. References for MDA (S Afr J Sci 2010;106:1-5) and glycogen determination (Arch Biochem 1950;25(1):191-200) are provided in the methods sections in the manuscript. Standard curves were used to determine the concentrations of the unknowns in all experiments and would not add value to the information on the figures unless provided as supplementary data. 5)The single IPGTT experiment using a 3 g/kg dose of sugar is inconclusive because the resulting measurements ~30 mmol/L fall outside the “dynamic range” of blood glucose homeostasis. As observed in Figure 5B, these animals lacking beta-cells cannot effectively respond to such doses of sugar, and do not return to baseline even after 2.5 hr. Therefore, lack of differences in this experiment are insufficient evidence to support the negative claim. The manuscript should be modified to take into account this possibility. Response: Whatever the amount of glucose administered, blood glucose concentrations never return to baselines in GTT even after 2 hrs in diabetic individuals and this is one of the criteria clinically used to diagnose diabetes. Fasting blood glucose concentrations in STZ-treated rats were above 25 mM before loading glucose anyway, meaning that any amount of glucose loaded would have spiked blood glucose levels beyond physiological range. In non-STZ-treated rats, 3.0 g/kg did not increase blood glucose concentrations beyond physiological ranges and there were no differences between controls and naringin-treated groups even in the presence of insulin suggesting lack of hypoglycemic effects of naringin in normal or low insulin concentrations. A lower dose of glucose could have produced a flat curves considering the differences in blood glucose concentrations between STZ-treated and non-STZ-treated rats. Hypoglycemic effects of naringin are reported in simulated type 2 diabetes models where there is hyperinsulinemia (Nutrients. 2013 Feb 27;5(3):637-50). _____
Additional Information:	

Question	Response
Financial Disclosure Please describe all sources of funding that have supported your work. A complete funding statement should do the following: Include **grant numbers and the URLs** of any funder's website. Use the full name, not acronyms, of funding institutions, and use initials to identify authors who received the funding. **Describe the role** of any sponsors or funders in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. If they had no role in any of the above, include this sentence at the end of your statement: "*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript*." If the study was **unfunded**, provide a statement that clearly indicates this, for example: "*The author(s) received no specific funding for this work*." * typeset	The study was funded by Career Development Award by the South African Medical Research Council to the corresponding author
Competing Interests You are responsible for recognizing and disclosing on behalf of all authors any competing interest that could be perceived to bias their work, acknowledging all financial support and any other relevant financial or non-financial competing interests. Do any authors of this manuscript have competing interests (as described in the PLOS Policy on Declaration and Evaluation of Competing Interests)? **If yes**, please provide details about any and all competing interests in the box below. Your response should begin with this statement: *I have read the journal's policy and the authors of this manuscript have the following competing interests:*	None to declare

If no authors have any competing interests to declare, please enter this statement in the box: "*The authors have declared that no competing interests exist.*" * typeset	
Ethics Statement You must provide an ethics statement if your study involved human participants, specimens or tissue samples, or vertebrate animals, embryos or tissues. All information entered here should **also be included in the Methods section** of your manuscript. Please write "N/A" if your study does not require an ethics statement. **Human Subject Research (involved human participants and/or tissue)** All research involving human participants must have been approved by the authors' Institutional Review Board (IRB) or an equivalent committee, and all clinical investigation must have been conducted according to the principles expressed in the Declaration of Helsinki. Informed consent, written or oral, should also have been obtained from the participants. If no consent was given, the reason must be explained (e.g. the data were analyzed anonymously) and reported. The form of consent (written/oral), or reason for lack of consent, should be indicated in the Methods section of your manuscript. Please enter the name of the IRB or Ethics Committee that approved this study in the space below. Include the approval number and/or a statement indicating approval of this research. **Animal Research (involved vertebrate animals, embryos or tissues)** All animal work must have been conducted according to relevant national and international guidelines. If your study involved non-human primates, you must provide details regarding animal welfare and steps taken to ameliorate suffering; this is in accordance with the	The study was conducted according to the guide lines of the Animal Ethics Committee of the University of KwaZulu-Natal that approved the study.

recommendations of the Weatherall report, "The use of non-human primates in research." The relevant guidelines followed and the committee that approved the study should be identified in the ethics statement. If anesthesia, euthanasia or any kind of animal sacrifice is part of the study, please include briefly in your statement which substances and/or methods were applied. Please enter the name of your Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board, and indicate whether they approved this research or granted a formal waiver of ethical approval. Also include an approval number if one was obtained. **Field Permit** Please indicate the name of the institution or the relevant body that granted permission.	
Data Availability PLOS journals require authors to make all data underlying the findings described in their manuscript fully available, without restriction and from the time of publication, with only rare exceptions to address legal and ethical concerns (see the PLOS Data Policy and FAQ for further details). When submitting a manuscript, authors must provide a Data Availability Statement that describes where the data underlying their manuscript can be found. Your answers to the following constitute your statement about data availability and will be included with the article in the event of publication. **Please note that simply stating 'data available on request from the author' is not acceptable.** ***If, however, your data are only available upon request from the author(s), you must answer "No" to the first question below, and explain your exceptional situation in the text box provided.*** Do the authors confirm that all data underlying the findings described in their manuscript are fully available without restriction?	Yes - all data are fully available without restriction
Please describe where your data may be	Data are contained within the paper

found, writing in full sentences. **Your answers should be entered into the box below and will be published in the form you provide them, if your manuscript is accepted.** If you are copying our sample text below, please ensure you replace any instances of XXX with the appropriate details. If your data are all contained within the paper and/or Supporting Information files, please state this in your answer below. For example, “All relevant data are within the paper and its Supporting Information files.” If your data are held or will be held in a public repository, include URLs, accession numbers or DOIs. For example, “All XXX files are available from the XXX database (accession number(s) XXX, XXX)." If this information will only be available after acceptance, please indicate this by ticking the box below. If neither of these applies but you are able to provide details of access elsewhere, with or without limitations, please do so in the box below. For example: “Data are available from the XXX Institutional Data Access / Ethics Committee for researchers who meet the criteria for access to confidential data.” “Data are from the XXX study whose authors may be contacted at XXX.” * typeset	
Additional data availability information:	

Cover Letter

Dear Editor,

It is my pleasure to submit our original research findings on the study we conducted to investigate the effects of naringin, a bioflavanoid on metabolic aspects of type 1 diabetes. Previous studies have suggested that naringin or its aglycone, naringenin have hypoglycemic effects and are associated with positive outcomes in experimental diabetes animal models. Contrary to some of these findings, our study seems to suggest that naringin does not exhibit hypoglycemic effects in insulin deficiency and may therefore, like metformin require insulin to lower blood glucose. We further report that naringin mitigates aspects of diabetic ketoacidosis in type 1 diabetes. We also report that naringin relieves oxidative stress associated with hyperglycemia in type 1 diabetes. The impact of our study would be that naringing supplements may prevent complications of type 1 diabetes such as ketoacidosis pending further clinical studies.

This is the first time we have submitted this manuscript to your journal and we do not have a preference for any academic editor, neither do we oppose any reviewers. The authors have declared that no competing interests exist.

Kind Regards,

Dr Owira PMO

Corresponding Authour.

GRAPEFRUIT-DERIVED FLAVONOID NARINGIN IMPROVES KETOACIDOSIS AND LIPID PEROXIDATION IN TYPE 1 DIABETES RAT MODEL

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Conflict of interest: None to declare

ABSTRACT

Background

Hypoglycemic effects of grapefruit juice are well known but the effects of naringin, its main flavonoid on glucose intolerance and metabolic complications in type 1 diabetes are not known.

Objectives

To investigate the effects of naringin on glucose intolerance, oxidative stress and ketonemia in type 1 diabetic rats.

Methods

Sprague-Dawley rats divided into 5 groups (n=7) were orally treated daily with 3.0 ml/kg body weight (BW)/day of distilled water (group 1) or 50 mg/kg BW of naringin (groups 2 and 4, respectively). Groups 3, 4 and 5 were given a single intra-peritoneal injection of 60 mg/kg BW of streptozotocin to induce diabetes. Group 3 was further treated with subcutaneous insulin (4.0 IU/kg BW) twice daily, respectively.

Results

Stretozotocin (STZ) only-treated groups exhibited hyperglycemia, polydipsia, polyuria, weight loss, glucose intolerance, low fasting plasma insulin and reduced hepatic glycogen content compared to the control group. Furthermore they had significantly elevated Malondialdehyde (MDA), acetoacetate, β-hydroxybutyrate, anion gap and significantly reduced blood pH and plasma bicarbonate compared to the control group. Naringin treatment significantly improved Fasting Plasma Insulin (FPI), hepatic glycogen content, malondialdehyde, β-hydroxybutyrate, acetoacetate, bicarbonate, blood pH and anion gap but not Fasting Blood Glucose (FBG) compared to the STZ only-treated group.

Conclusions

Naringin is not hypoglycemic but ameliorates ketoacidosis and oxidative stress. Naringin supplements could therefore mitigate complications of diabetic ketoacidosis.

Key words

Naringin, antioxidants, ketoacidosis, hyperglycemia.

INTRODUCTION

Diabetes mellitus (DM) is a consequence or absolute of relative insulin deficiency leading to hyperglycemia and concomitant disturbances in carbohydrate, fat and protein metabolism [1, 2].

Diabetic ketoacidosis (DKA) is an acute life threatening complication of DM. It is defined by blood glucose >11 mmol/l, venous pH <7.3, and bicarbonate <15 mM, ketonemia and ketonuria [3, 4]. DKA primarily affects patients with type 1 but can also occur in type 2 diabetes under conditions of metabolic stress such as infection, trauma and surgery [5].

Hyperglycemia-induced oxidative stress causes pancreatic β-cell dysfunction due to pro-inflammatory cytokines which induce the release of insulin counter-regulatory hormones (glucagon, cortisol and growth hormone) leading to increased hepatic gluconeogenesis and hyperglycemia [6, 7, 8]. Increased lipolysis accelerate the delivery of free fatty acids to the liver for ketone body {acetoacetate (AcAc) and β-hydroxybutyrate (3-HB)} synthesis [7, 9]. AcAc and 3-HB are strong organic anions that dissociate freely generating increased hydrogen ions which overwhelm the normal plasma bicarbonate buffering capacity resulting in metabolic acidosis and increased anion gap (AG), (defined as the sum of serum chloride and bicarbonate concentrations subtracted from the serum sodium concentrations) [9, 10, 11].

Therapeutic management of DKA is yet to be optimised but includes adequate fluid replacement and insulin infusion to correct electrolyte imbalance and hyperglycaemia, respectively [4]. Currently, there are no clear-cut guidelines defining loss of glycemic control or propensity to hyperosmolar states (blood glucose of 33 mM or more) which may predispose vulnerable patients to DKA. However, monitoring of sodium, potassium, magnesium and phosphorus levels to maintain electrolyte balance, hemogram to assess anaemia and blood pH and gas analysis to determine ketonemia are routinely done. Contentious issues on fluid replacement therapy include the amount and type of fluids (normal saline or Ringer's lactate) to be used and the rate of delivery [4, 12]. Routinely, normal saline is used for fluid expansion followed by intravenous insulin infusion at 0.1 U/kg/h until the patient is stabilised with dextrose to maintain euglycemia then switched to subcutaneous insulin with a dietary plan [12]. However, hospitalisation, stabilisation and subsequent follow-up pose challenges especially to patients with disadvantaged socio-economic backgrounds hence a dietary plan that mitigates the onset of DKA may be a viable cost effective patient care option.

Consequently, medicinal plants which have traditionally been used to manage diabetes offer some hope as they have less side-effects commonly associated with conventional medications [9]. Naringin (4',5,7-trihydroxy flavonone-7-rhamnoglucoside), the major flavonoid in grapefruit juices has been shown to possess pharmacological properties such as antioxidant, antidiabetic and antidyslipidemic effects [13, 14, 15, 16]. Hypoglycemic effects of naringin are well documented [17, 18] and Punithavathi et al [15] have further shown that 30 mg/kg of naringin co-administered with 50 mg/kg of vitamin C prevented oxidative stress, improved fasting plasma insulin concentrations and glucose intolerance. Hyperglycemia in a diabetic state is associated with increased oxidative stress [15, 16] which exacerbates DKA hence it is envisaged that with its demonstrated antioxidant effects, naringin could ameliorate glucose intolerance and metabolic complications associated with DKA.

Aims and objectives

This study was designed to investigate the effects of naringin on glucose intolerance, metabolic benchmarks of DKA and oxidative stress in streptozotocin (STZ)-induced diabetes in rats.

MATERIAL AND METHODS

Chemicals and Reagents

Naringin, D-glucose, STZ, citrate buffer, hydrochloric acid, sulphuric acid, potassium hydroxide, ethanol, sodium sulphate and phenol were all purchased from Sigma-Aldrich Pty. Ltd, Johannesburg, South Africa.

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 Resource Unit (BRU) of the University of KwaZulu-Natal, Durban, South Africa.

Animal Treatment

Male Sprague-Dawley rats of 200-300g body weight were provided by the Biomedical Resource Unit of the University of KwaZulu-Natal and divided into 5 groups (n = 7), 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 which approved the study.

Type 1 DM was induced by a single intraperitoneal injection of 60 mg/kg BW of STZ dissolved in 0.2 ml citrate buffer, pH 4.5 after an overnight fast in groups 3, 4 and 5, respectively. Diabetic

status was confirmed 3 days after STZ administration by tail pricking to analyse the blood glucose levels. Animals with random blood glucose concentrations above 11.0 mmol/L were considered diabetic and were included in the study [19] and immediately commenced on treatment.

Naringin (50 mg/kg BW/day in distilled water) was orally administered to groups 2 and 4 while regular insulin (4.0 IU/kg) was further administered subcutaneously twice daily to group 3. Distilled water (3.0 ml/kg BW/day) was administered to groups 1 and 5, respectively orally, (Table 1). Animal weights and water consumption were measured daily. The animals were further placed in solitary metabolic cages on day 40 of treatment and 24-hour urine samples collected measured and recorded. The animals were sacrificed by halothane overdose on day 42 of treatment and blood samples collected via cardiac puncture and plasma samples stored at -80°C for further biochemical analysis.

Table 1. Animal treatment protocol. The animals were weighed and randomly divided into 5 groups, (n=7). Naringin and distilled water were orally administered daily. BW = per kg body weight.

Treatment / Groups	**Distilled H_2O, (ml/BW) (Normal control)**	**Naringin, (mg/BW) (Normal)**	**Insulin, (U/ BW SC, b.d) (Diabetic)**	**Naringin, (mg/BW) (Diabetic)**	**Distilled H_2O, (ml/BW) (Control Diabetic)**
1	3.0	-	-	-	-
2	-	50.0	-	-	-
3	-	-	4.0	-	-
4	-	-	-	50.0	-
5	-	-	-	-	3.0

2.3 **Methods**

2.3.1 **Blood glucose testing**

FBG measurements were done on treatment days 0, 14 and 28, respectively. On treatment day 41, Glucose Tolerance Tests (GTT) were done after an overnight fast followed by a single intraperitoneal injection of 3.0 mg/kg BW of glucose in normal saline and blood glucose levels were measured at times 0, 15, 30, 60, 90 and 120 minutes. Insulin treatment for group 4 was withheld on the day of GTT. Blood glucose analysis was done as previously described and the calculated Area under the curve (AUC) from blood-glucose concentration-time curves presented as AUC units {(mmol/L) X Time (minutes)}.

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. The optical density was determined by a microplate reader (EZ Read 400, Biochrom®) at 450 nm.

Hepatic glycogen assay

Hepatic glycogen content was measured by the modified method of Seifter *et al.*,[20]. Briefly, the liver tissue was homogenised in 1.0 ml of 30% KOH saturated with Na_2SO4. The homogenate obtained was dissolved by boiling in a water bath (100˚C) for 30 minutes, vortexed and cooled on ice. Glycogen was then precipitated with 2.0 ml of 95% ethanol, vortexed, incubated on ice for 30 min and later centrifuged at 550 g for 30 min. The glycogen pellets obtained were then re-dissolved in 1.0 ml of distilled water which was thereafter treated with 1.0 ml of 5% phenol and 5 ml of 96-98% sulphuric acid respectively. This was incubated on 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.

153 **Determination of serum electrolyte and blood pH levels**

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

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

159 $AG = \{[Na^+] + [K^+]\} - \{[Cl^-] + [HC0^{3-}]$ [11]

160 **Plasma thiobarbituric acid reactive substances (TBARS) assay**

161 Plasma TBARS assay was carried out according to the modified method of Phulukdaree *et al*
162 [21])**.** Briefly, 200 µl of plasma samples were added to 500 µl of 2% phosphoric acid (H_3PO_4),
163 400 µl of 7% H_3PO_4 and 400 µl of BHT/TBA solution in a set of clean glass test-tubes. In
164 another set of eight clean fresh test tubes, 200 µl of serially diluted malondialdehyde (MDA)
165 standard was added to 500 µl of 2% H_3PO_4, 400 µl of 7% H_3PO_4 and 400 µl of butylated
166 hydroxytoluene (BHT)/ Thiobarbituric acid (TBA) solution. The reactions in both sets of tubes
167 were initiated with 200 µl of 1.0 M HCl. All tubes were incubated in a shaking boiling water
168 bath (100^{o}C) for 15 minutes and cooled at room temperature. n-butanol (1.5 ml) was then added
169 to each tube and mixed thoroughly. Top phase (200 µl) was then transferred to a 96-well micro-
170 plate in and read at 532 and 600 nm using micro-plate reader (Spectrostar®). Plasma MDA
171 concentrations were calculated using an extinction coefficient of $1.56x10^5 M^{-1}cm^{-1}$.

172 **Analysis of serum ketone body concentrations**

173 Serum and urine ketone body levels were determined by the spectrophotometric enzymatic
174 assay kit (Enzychrom ™, BioAssay systems, EKBD-100) according to the manufacturer's

instructions. The optical density (OD) was read at 340 nm using a spectrophotometer (Genesys 20, Thermospectronic) and the concentrations calculated according to the formula:

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

A similar procedure was repeated for the 3-β hydroxybutyrate (3-HB) assay using the 3-HB buffer, reagent and standard, respectively and the concentrations calculated using the formula:

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

Statistics

The data was presented as mean ± SD and analyzed by GraphPad Prism Software® Version 5.0. One-Way ANOVA or Student t-tests and non-parametric Mann–Whitney tests were used where applicable to determine statistical significance. Values of $P < 0.05$ were taken to imply statistical significance.

RESULTS

Animal growth change during treatment period

STZ only-treated groups exhibited significant ($p<0.0001$) weight loss compared to the normal control group. Treatment with insulin or naringin significantly ($p<0.0001$) increased weight gain compared to the non-treated STZ group. Treatment with naringin had no significant change in weight gain in normal rats compared to the control (Fig 1).

Figure 1: Animal weight changes during treatment period. (***$p<0.0001$ compared to normal control, #$p<0.0001$ compared to non-treated STZ group).

Water consumption during treatment period

The average daily water consumption was significantly ($p<0.0001$) higher in untreated STZ rats compared to normal control group. However, water intake was significantly ($p<0.0001$) reduced in the insulin or naringin treated groups compared to the non-treated STZ group (Fig 2).

Fig 2. Water consumption during treatment period. (***$p<0.0001$ compared to normal control, #$p<0.0001$ compared to untreated STZ group).

Urine output

The urine output was significantly ($p<0.0001$) elevated in the untreated STZ group in comparison to the normal control group. Treatment with insulin significantly ($p<0.05$) reduced urine output in STZ treated animals compared to the untreated STZ group. However, naringin treatment did not have any significant effect on urine output in STZ-treated animals compared to the untreated STZ group (Fig 3).

Figure 3. 24-hour urine output collected after the animals were put in metabolic cages. (***$p<0.0001$ compared to normal control, # $p<0.05$ compared to untreated STZ group).

Glucose tolerance

FBG levels were significantly higher ($p<0.0001$) in the untreated STZ group compared to the normal control group. However, insulin but not naringin treatment significantly ($p<0.05$) improved the FBG levels compared to the untreated STZ group (Fig 4).

Fig 4. Fasting blood glucose levels measured by tail pricking after an overnight fast. (***$p<0.001$ compared to the normal control group, #$p<0.05$ compared to untreated STZ group).

The untreated STZ group showed glucose intolerance compared to the normal control (Fig 5A and B) with significantly ($p<0.0001$) increased calculated Area Under the Curve (AUC) further

compared to normal control group. Neither naringin nor insulin treatment significantly improved the AUCs in STZ-treated group (Figure 5C).

Fig 5. Glucose Tolerance Tests (GTT) after the animals were fasted overnight and challenged with intreperitoneal injection of 3.0 g/kg BW of glucose in normal saline in normal animals treated with distilled water (controls) or naringin (**A**) and STZ-treated animals (**B**). Insulin treatment was withheld on the day of GTT and the figures were plotted in different graphs for clarity considering the large differences in blood glucose concentrations between normal and STZ groups on the y-axis. **C**; Calculated AUC from GTT. (***$p<0.0001$ compared to the normal control group).

Fasting plasma insulin

FPI concentration were significantly ($p<0.0001$) lower in the STZ-treated group compared to the normal control group. Naringin or insulin significantly ($P<0.01$) improved FPI concentrations in the STZ-treated compared to the untreated STZ group, respectively (Fig 6). Surprisingly, naringin treatment significantly increased FPI concentrations in normal rats compared to the controls.

Fig 6. Plasma insulin concentrations after blood was collected by cardiac puncture. (***$p<0.0001$, ^$p<0.05$ compared to normal control, #$p<0.05$ compared to STZ only-treated animals).

Hepatic glycogen levels

Hepatic glycogen concentrations were significantly ($p<0.0001$) reduced in STZ-treated compared to the control group. Treatment with insulin or naringin significantly ($p<0.05$)

increased hepatic glycogen content compared to the STZ only-treated group, respectively. Naringin treatment of normal rats caused significant ($p<0.05$) elevation of hepatic glycogen content compared to the normal control (Fig 7).

Fig 7. Glycogen levels in homogenised hepatic tissue. (***$p< 0.0001$, ^$p< 0.05$ compared to normal control, #$p<0.05$ compared to STZ only-treated rats, respectively)

Plasma lipid peroxidation (TBARS assay)

Plasma MDA concentrations in STZ only-trated group were significantly ($p<0.0001$) elevated compared to the normal control group. Naringin or insulin significantly ($p<0.0001$) reduced plasma MDA concentrations in STZ-trated rats compared to the STZ only treated group, respectively (Fig 8).

Fig 8. Plasma concentrations of MDA measured as a marker for lipid peroxidation. (***$p<0.001$ compared to normal control, #$p<0.0001$ compared to STZ only-treated group).

Serum β-hydroxybutyrate (3-HB) levels

Serum β-hydroxybutyrate (3-HB) levels were significantly ($p<0.001$) elevated in the STZ only-treated compared to the normal control group. Treatment with insulin or naringin significantly ($p<0.05$) reduced the levels of 3-HB compared to the STZ only-treated group, respectively (Fig 9A).

Serum acetoacetate (AcAc) levels

Serum acetoacetate (AcAc) levels were significantly ($p<0.0001$) elevated in the STZ only-teated compared to normal control group. However, naringin significantly ($p<0.05$) reduced plasma AcAc levels in STZ-treated compared to the STZ only-treated rats (Fig 9B).

Fig 9. **A**; Serum 3-β-hydroxybutyrate (3HB) levels. (***p<0.0001 compared to normal untreated control, #p<0.05 compared to STZ only-treated rats) and **B**; Serum acetoacetate (AcAc) levels. (***p<0.0001 compared to normal control, #p<0.05 compared to STZ only-treated group)

Serum electrolyte and blood pH

Serum sodium, chloride and bicarbonate and blood pH were significantly (p<0.01, p<0.0001, p<0.0001 and p<0.05, respectively) reduced, while potassium was significantly (p<0.05) elevated in the STZ only-treated compared to the normal control group. Insulin significantly (p<0.05) increased sodium and reduced potassium in the STZ-treated compared to the STZ only-treated group, respectively. Naringin or insulin significantly (p<0.05) increased plasma bicarbonate and blood pH in STZ-treated compared to the STZ only-treated diabetic rats, respectively (Table 2).

Table 2. Serum electrolytes and blood pH. (***p<0.0001, **p<0.01 and *p<0.05 compared to the normal control group, $^{\#}$p<0.0001, $^{@}$p<0.01 and $^{\&}$p<0.05 compared to the STZ only-treated group).

	Normal control	**Normal naringin**	**STZ-insulin**	**STZ-Naringin**	**STZ**
Sodium (mM)	142.70±0.56	142.80±0.37	140.00±1.00**&**	132.20±1.50	135.00±1.79****
Potassium (mM)	6.42±0.30	7.120±0.59	5.87±0.12**&**	7.34±0.59	7.56±0.46*
Chloride (mM)	103.80±0.89	103.00±0.63	100.30±1.33	96.20±2.40	96.40±1.36*****
Bicarbonate (mM)	24.77±0.76	24.74±1.04	21.18±0.69 #	17.63±0.64 **@**	13.10±0.54 *****
Blood pH	7.4±0.24	7.33±0.20	7.33±0.17**&**	7.4.00±0.31**@**	6.64±0.30****

Anion gap (AG)

Calculated AG was significantly ($p<0.0001$) increased in the STZ only-treated group compared to the normal control group. Naringin or insulin treatment significantly ($p<0.05$) decreased the serum AG in the STZ-treated groups compared to the STZ only-treated group, respectively (Fig 10).

Fig 10: Anion gap (AG) calculated from serum electrolytes as previously described [11]. ($^{***}p< 0.0001$ compared to normal control group, $^{\#}p< 0.05$ compared to the STZ only-treated group).

DISCUSSION

This study was based on a typical type 1 diabetes model created with STZ which selectively and irreversibly completely destroys the pancreatic β-cells by oxidative damage. Interestingly, naringin treatment of normal rats was associated with relative increase in FPI concentrations (Fig 6). Considering that insulin treatment similarly increased FPI in diabetic animals, we speculate that the antioxidant effects of naringin reduced oxidative stress in the pancreatic β-cell mitochondria leading to increased ATP synthesis and subsequent insulin secretion. By its anabolic effects, insulin similarly to naringin, boosted residual anti-oxidant capacity of the β-cells leading to increased insulin secretion. This could have limited oxidative damage of STZ to allow the β-cells to retain some residual activity. Potential insulinotropic effects of naringin have only recently been demonstrated [22].

STZ-treated rats exhibited significant weight loss compared to controls (Fig 1). It may be argued that diabetes conferred hypophagic state since we did not measure food intake but it should also be considered that loss of body mass is a well known phenomenon in type 1 diabetes irrespective of food consumption rates [3, 13, 15]. That naringin, like insulin significantly attenuated weight loss in diabetic rats further suggests that, weight loss in diabetic

rats was due to lipid and protein catabolism (associated with insulin deficiency) which were inhibited by both naringin and insulin, respectively. Hence we speculate that antioxidant effects of naringin reduced oxidative stress and free radicals which are known to provoke the release of catabolic insulin counter-regulatory hormones [9].

Polydipsia and polyuria were also observed in STZ-treated animals compared to the normal control group (Fig 2 and 3). Hyperglycemia in DKA leads to increased extracellular osmolality which causes dehydration. As a compensatory mechanism, increased osmolality activates hypothalamic osmoreceptors causing the release of antidiuretic hormone (ADH) which corrects the hyperosmolar state [23]. Failure of this mechanism leads to activation of thirst leading to the increased water intake. Insulin reversed both polyuria and polydipsia in STZ-treated rats. However, naringin treatment reversed only polydipsia but not polyuria suggesting that naringin inhibited thirst activation but had no anti-diuretic effects either in the normal or STZ-treated rats.

Unlike insulin, naringin improved neither FBG nor glucose intolerance in STZ-treated rats (Fig 4). We previously reported that naringin does not ameliorate hyperglycemia in diabetic type 1 rats [13]). This is contrary to other studies that have reported hypoglycemic effects of naringin and its aglycone naringenin to be mediated by suppression of hepatic expression of key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [13-15, 17, 24]. We concur with these observations but opine that these studies were done in simulated type 2 diabetes models where there was insulin resistance but not absolute deficiency. That insulin treatment significantly improved fasting blood glucose and plasma insulin, respectively suggests that by its anabolic effects insulin could have promoted modest pancreatic β-cell recovery in STZ- treated animals, enhanced residual insulin secretory capacity or both (Fig 4, 5B and 6). Glucose tolerance was similar between naringin-treated and control rats but calculated AUC suggested that STZ-treated rats were significantly

glucose intolerant compared to normal controls (Fig 5). GTT was conducted with intraperitoneal injections of 3.0 mg/kg body weight of glucose in normal saline and even though we could not measure concurrent plasma insulin response, our results suggest that STz-treated rats were already in hyperosmolar state considering the high fasting blood glucose concentrations (Fig 4 and 5 B). Since insulin treatment was withheld during GTT, it is therefore not surprising that glucose tolerance did not improve in the insulin-treated group compared to STZ only-treated group.

Liver glycogen content was significantly reduced in STZ only-treated rats compared to controls but this was significantly reversed by either naringin or insulin treatment (Fig 7). This suggests that in the absence of insulin, glucagon and other insulin counter-regulatory hormones promoted gluconeogenesis and glycogenolysis which reduced glycogen storage in the liver in STZ-treated rats but this was inhibited by either naringin or insulin treatment. Naringin treatment of normal rats interestingly significantly increased hepatic glycogen content compared the normal controls (Fig 7). Insulin is known to activate glycogen storage by increasing expression of glycogen synthase which catalyses the rate-limiting step in glycogen synthesis [25]. Even though our study did not investigate the direct effects of naringin on hepatic expression of glycogen synthase, previous studies have shown that naringin or its aglycone naringenin suppresses PEPCK and G6Pase activities via activation of AMP-activated protein kinase (AMPK) [26, 27]. It is therefore likely that in our study, naringin failed to exert hypoglyecemic effects due to insulin deficiency and hence argue that naringin has metformin-like effects.

Oxidative stress causes lipid peroxidation due to increased Reactive Oxygen Species (ROS) leading to overproduction of MDA, which is used as a biomarker [28, 29]. Lipid peroxidation was significantly elevated in the STZ-treated compared to the control group but was significantly reversed by naringin treatment (Fig 8). Oxidative stress stimulates production of

pro-inflammatory cytokines which cause the release of insulin counter-regulatory hormones that are known to promote ketoacidosis [3]. Therefore, the antioxidant effects of naringin either directly or through enhancing antioxidant defence systems directly or by augmenting insulin secretion reversed ketoacidosis. This is demonstrated by the significantly reduced plasma 3-HB and AcAc in diabetic rats that were treated with naringin compared to the untreated diabetic group (Fig 9A and B). Insulin significantly reduced plasma 3-HB compared to untreated diabetic animals suggesting that naringin like insulin could be having a direct inhibitory effect on hydroxymethyl glutayryl Coenzyme A (HMGCoA) synthase, which catalyses the rate-limiting step in ketogenesis [9].

Jung et al., [25] previously showed that naringin suppresses plasma carnitine palmitoyl-O-transferase (CPT) which is involved in the transport of free fatty acids (FFA) across the mitochondrial membrane for β-oxidation and ketogenesis. Therefore, with reduced CPT levels, there is reduced ketogenesis due to decreased supply of FFA. Increased ketogenesis observed in diabetes is associated with overproduction and accumulation of ketone bodies which are strong organic anions. These dissociate freely producing hydrogen ions which bind and overwhelm serum bicarbonate buffering capacity eventually leading to metabolic acidosis in diabetes. However, naringin significantly improved plasma bicarbonate and blood pH levels, compared to STZ only-treated group (Table 2). Subsequently acid-base imbalance marked by increased AG was significantly reversed by naringin rats compared to STZ only-treated group (Fig 10). However, unlike insulin, naringin did not correct electrolyte imbalance in diabetic animals suggesting that like with insulin treatment, fluid replacement therapy should precede naringin supplementation were it to be used clinically. These observations confirm that naringin reversed metabolic acidosis associated with DKA albeit in experimental animal model. We recently reported antidiabetic effects of grapefruit juice in experimental animal studies [30]. Naringin is the main flavanoid in grapefruit juice and therefore it seems plausible

to infer that some of the antidiabetic effects of grapefruit juice could be attributed to naringin in addition to other as yet unidentified bioactive chemical ingredients. However, naringin on its own appears in this study to be associated with favourable metabolic end-points in type 1 diabetes. There is no known toxicity of naringin both to humans or experimental laboratory animals [31, 32] and we used pharmacologically effective 50 mg/kg body of naringin [13, 33, 34] owing to its poor solubility in water which was dose limiting by oral gavage. Considering the failure of known endogenous antioxidants like vitamins E and C to exert positive pharmacological effects on degenerative metabolic diseases in large scale clinical trials [32], our results presented here suggest that citrus-fruit-derived flavanoids like naringin could be viable cheaper alternatives.

Conclusion.

Although naringin did not improve glucose intolerance, it was associated with reversal of weight loss, improved glycogen storage and insulin secretion in diabetic rats. However, naringin abrogated metabolic acidosis suggesting a role in the management of DKA. These actions of naringin appear to be mediated in part by its powerful antioxidant affects. Our findings therefore suggest that naringin as a nutritional supplement could be protective against ketonemia in type 1 diabetes patients and may be used as adjunct therapy pending further clinical studies.

Acknowledgements:

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Figure 1

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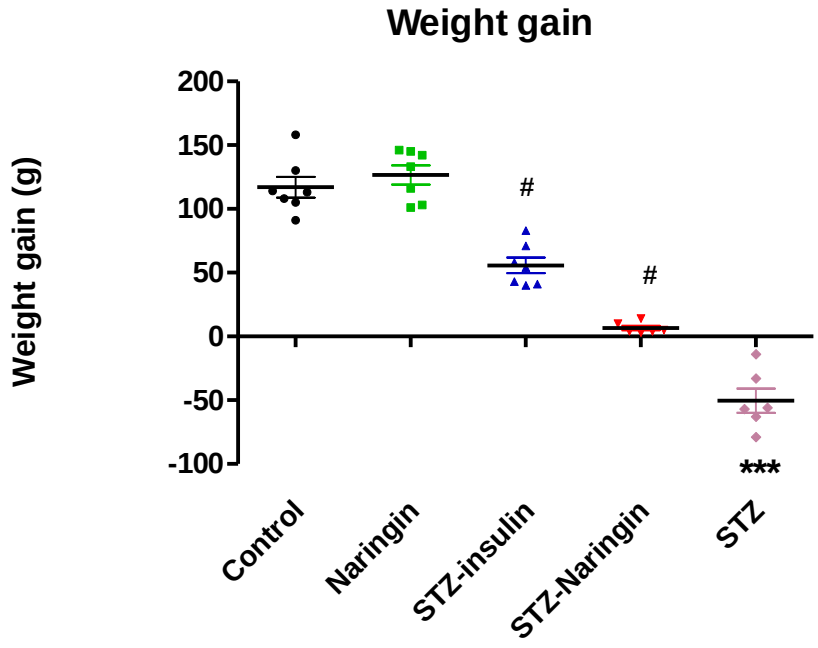

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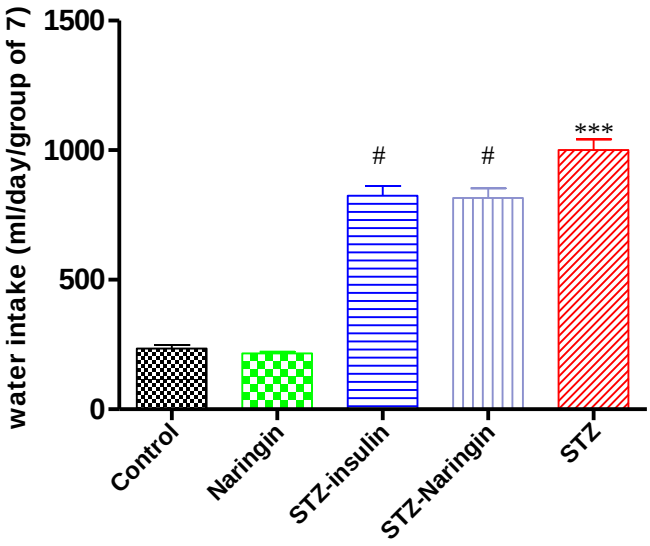

Figure 3

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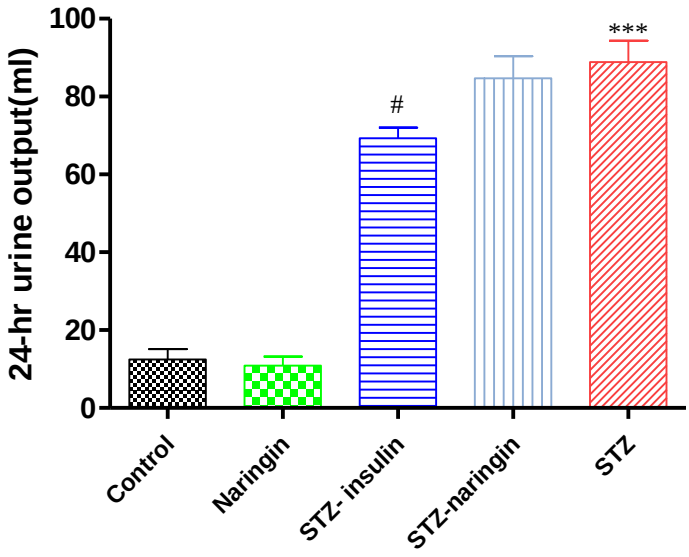

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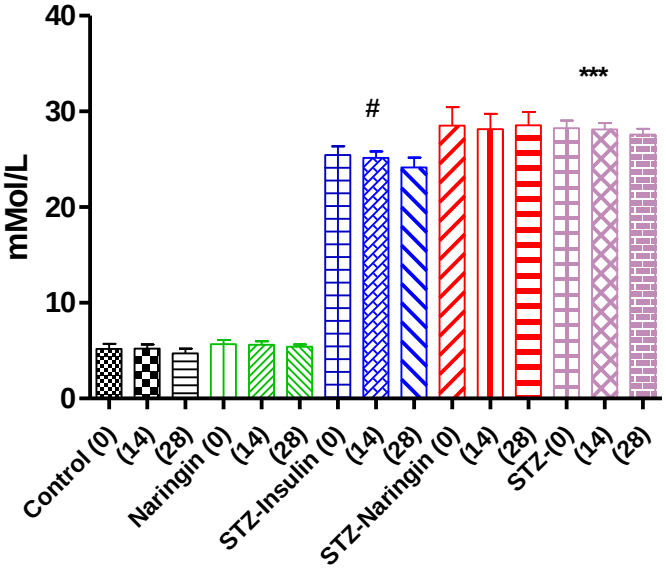

Figure 5

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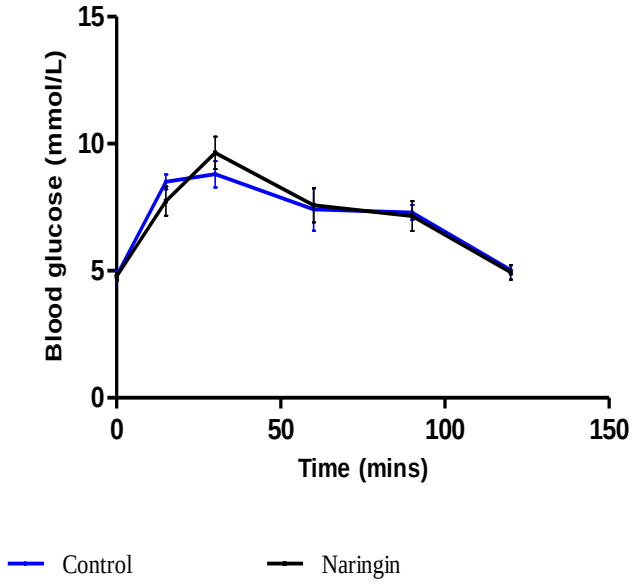

Control Naringin

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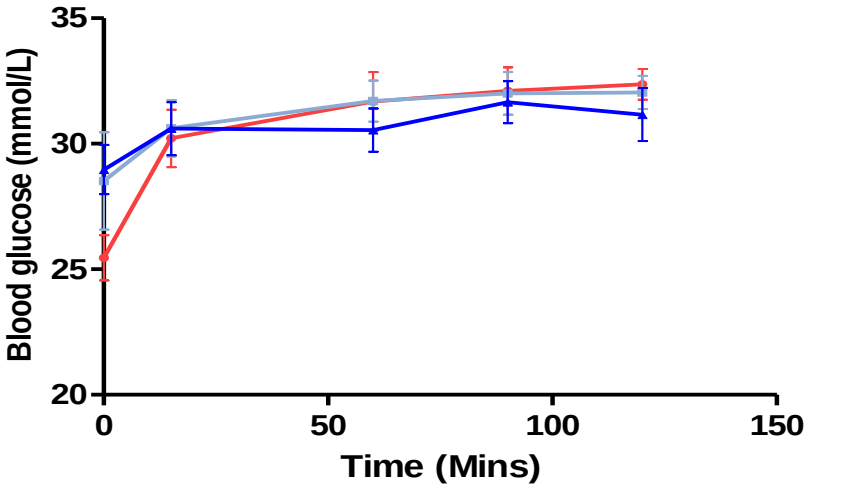

STZ- insulin STZ-Naringin STZ

C

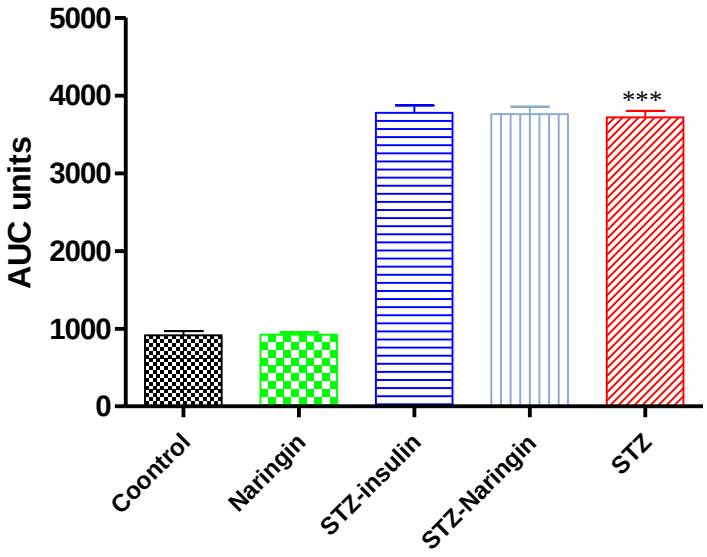
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Figure 6

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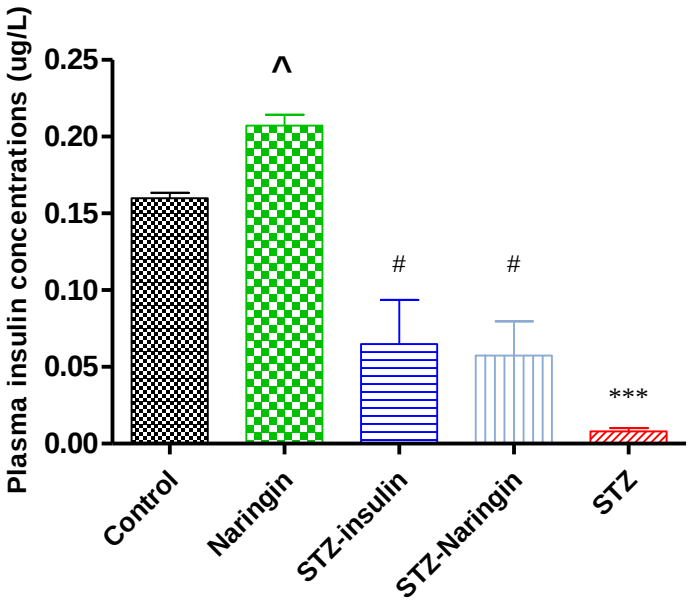

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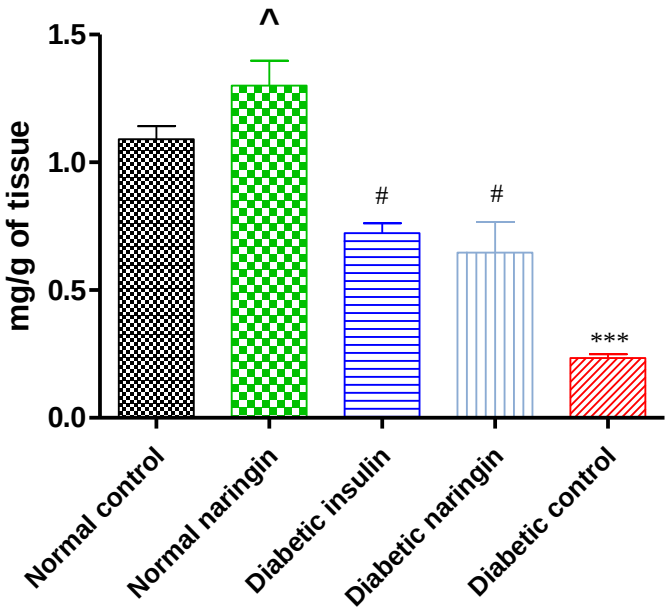

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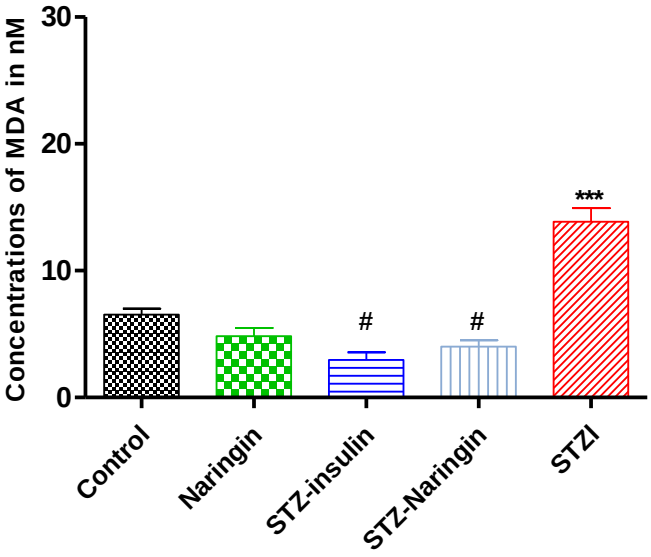

Figure 9

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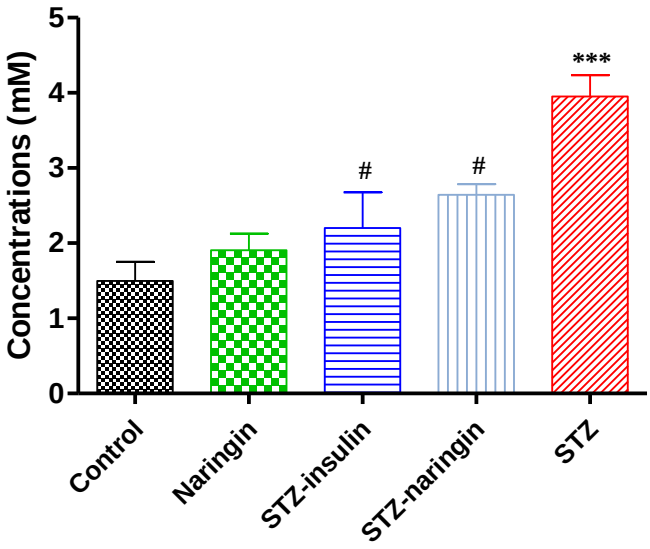

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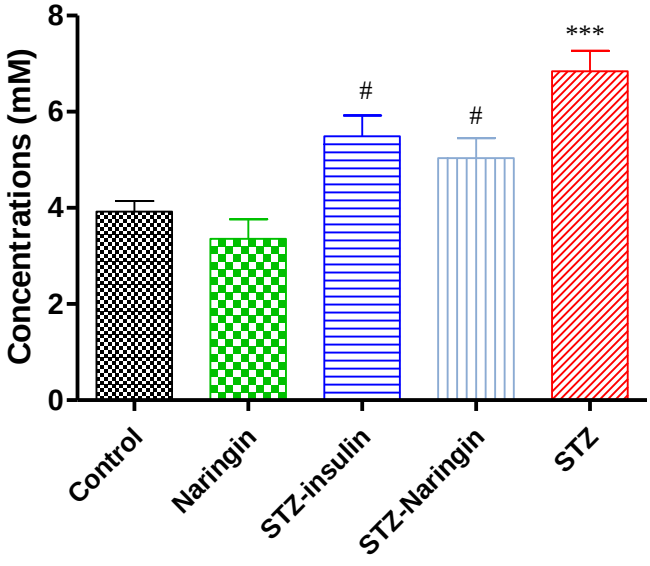

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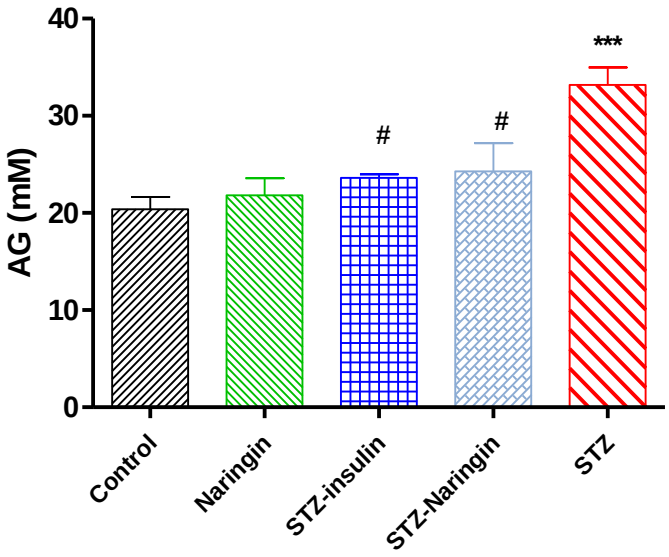

GRAPEFRUIT-DERIVED FLAVONOID NARINGIN ~~DOES NOT~~ IMPROVES KETOACIDOSIS AND LIPID PEROXIDATION IN TYPE 1 DIABETES RAT MODEL ~~GLUCOSE INTOLERANCE BUT MITIGATES OXIDATIVE STRESS AND KETOACIDOSIS IN TYPE 1 DIABETIC RATS~~

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Conflict of interest: None to declare

ABSTRACT

Background

Hypoglycemic effects of grapefruit juice are well known but the effects of naringin, its main flavonoid on glucose intolerance and metabolic complications in type 1 diabetes are not known.

Objectives

To investigate the effects of naringin on glucose intolerance, oxidative stress and ketonemia in type 1 diabetic rats.

Methods

Sprague-Dawley rats divided into 5 groups (n=7) were orally treated daily with 3.0 ml/kg body weight (BW)~~-~~/day of distilled water (group 1) or 50 mg/kg BW of naringin (groups 2 and 4, respectively). Groups 3, 4 and 5 were given a single intra-peritoneal injection of 60 mg/kg BW of streptozotocin to induce diabetes. Group 3 was further treated with subcutaneous insulin (4.0 IU/kg BW) twice daily, respectively.

Results

~~Untreated diabetic~~Stretozotocin (STZ) only-treated groups exhibited hyperglycemia, polydipsia, polyuria, weight loss, glucose intolerance, low fasting plasma insulin and reduced hepatic glycogen content compared to the control group. ~~The diabetic groups~~Furthermore they ~~also~~ had significantly elevated Malondialdehyde (MDA), acetoacetate, β-hydroxybutyrate, anion gap and significantly reduced blood pH and plasma bicarbonate compared to the control group. Naringin treatment significantly improved Fasting Plasma Insulin (FPI), hepatic glycogen content, malondialdehyde, β-hydroxybutyrate~~-~~, acetoacetate~~-~~, bicarbonate, blood pH

and anion gap but not ~~fasting~~ Fasting ~~blood~~ Blood ~~glucose~~ Glucose (FBG) compared to the ~~untreated diab~~STZ only-treated ~~etic~~ group.

Conclusions

Naringin is not hypoglycemic but ameliorates ketoacidosis and oxidative stress. Naringin supplements could therefore mitigate complications of diabetic ketoacidosis.

Key words

Naringin, antioxidants, ketoacidosis, hyperglycemia.

INTRODUCTION

Diabetes mellitus (DM) is a consequence or absolute of relative insulin deficiency leading to hyperglycemia and concomitant disturbances in carbohydrate, fat and protein metabolism [1, 2].

Diabetic ketoacidosis (DKA) is an acute life threatening complication of DM. It is defined by blood glucose >11 mmol/l, venous pH <7.3, and bicarbonate <15 mM, ketonemia and ketonuria [3, 4]. DKA primarily affects patients with type 1 but can also occur in type 2 diabetes under conditions of metabolic stress such as infection, trauma and surgery [5].

~~Concomitant increase in insulin counter-regulatory hormones such as glucagon, catecholamines, cortisol and growth hormone are risk factors for DKA [6].~~ Hyperglycemia-induced oxidative stress ~~exacerbates~~ causes pancreatic β-cell dysfunction ~~courtesy of~~due to pro-inflammatory cytokines which ~~further exacerbate~~induce the release of ~~pro-hyperglycemia~~ insulin counter-regulatory hormones~~-~~ (glucagon, cortisol and growth hormone) ~~[7]~~leading to increased ~~. The catabolic nature of these hormones antagonises insulin action in the liver, adipose and skeletal tissues leading to~~ hepatic gluconeogenesis and hyperglycemia [6, ~~8~~7, ~~9~~8].

Increased lipolysis accelerate the delivery of free fatty acids to the liver for ketone body {acetoacetate (AcAc) and β-hydroxybutyrate (3-HB)} synthesis [~~8~~7, ~~10~~9]. AcAc and 3-HB are strong organic anions that dissociate freely generating increased hydrogen ions which overwhelm the normal plasma bicarbonate buffering capacity resulting in metabolic acidosis and increased anion gap (AG), (defined as the sum of serum chloride and bicarbonate concentrations subtracted from the serum sodium concentrations) [~~10~~9, ~~11~~10, ~~12~~11].

~~Life threatening cerebral oedema is a major complication of DKA and is the most common cause of diabetes-related deaths in children or foetal loss in pregnant diabetic patients [3, 13]. Concurrent infections, poor compliance to insulin treatment, drugs that affect carbohydrate metabolism or acute illnesses such as pancreatitis and myocardial infarction are further precipitating factors [14, 15].~~

Consequently, medicinal plants which have traditionally been used to manage diabetes offer some hope as ~~their consumption is no longer for nutritive purposes only but also as nutraceuticals devoid of adverse effect~~they have less~~s~~ side-effects commonly associated with conventional medications [~~10~~9]. Naringin (4',5,7-trihydroxy flavonone-7-rhamnoglucoside), the major ~~flavanoid~~ flavonoid in grapefruit juices has been shown to possess pharmacological properties such as antioxidant, antidiabetic and antidyslipidemic effects [~~17~~13, ~~18~~14, ~~19~~15, ~~20~~16]. Hypoglycemic effects of naringin are well documented [~~21~~17, ~~22~~18] and Punithavathi et al [~~19~~15] have further shown that 30 mg/kg of naringin co-administered with 50 mg/kg of vitamin C prevented oxidative stress, improved fasting plasma insulin concentrations and glucose intolerance. Hyperglycemia in a diabetic state is associated with increased oxidative stress [~~19~~15, ~~20~~16] which exacerbates DKA hence it is envisaged that with its demonstrated ~~powerful~~ antioxidant effects, naringin could ameliorate glucose intolerance and metabolic complications associated with DKA.

Aims and objectives

This study was designed to investigate the effects of naringin on glucose intolerance, metabolic benchmarks of DKA and oxidative stress in streptozotocin (STZ)-induced diabetes in rats.

MATERIAL AND METHODS

Chemicals and Reagents

Naringin, D-glucose, STZ, citrate buffer, hydrochloric acid, sulphuric acid, potassium hydroxide, ethanol, sodium sulphate and phenol were all purchased from Sigma-Aldrich Pty. Ltd, Johannesburg, South Africa.

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 Resource Unit (BRU) of the University of KwaZulu-Natal, Durban, South Africa.

Animal Treatment

Male Sprague-Dawley rats of 200-300g body weight were provided by the Biomedical Resource Unit of the University of KwaZulu-Natal and divided into 5 groups (n = 7) ~~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 which approved the study.

Type 1 DM was induced by a single intraperitoneal injection of 60 mg/kg BW of STZ dissolved in 0.2 ml citrate buffer, pH 4.5 after an overnight fast in groups 3, 4 and 5, respectively. Diabetic

status was confirmed 3 days after STZ administration by tail pricking to analyse the blood glucose levels. Animals with random blood glucose concentrations above 11.0 mmol/L were considered diabetic and were included in the study [~~23~~19] and immediately commenced on treatment.

Naringin (50 mg/kg BW/day in distilled water) was orally administered to groups 2 and 4 while regular insulin (4.0 IU/kg) was further administered subcutaneously twice daily to group 3. Distilled water (3.0 ml/kg BW/day) was administered to groups 1 and 5, respectively orally, (Table I). Animal weights and water consumption were measured daily. The animals were further placed in solitary metabolic cages on day 40 of treatment and 24-hour urine samples collected measured and recorded. The animals were sacrificed by halothane overdose on day 42 of treatment and blood samples collected via cardiac puncture and plasma samples stored at -80°C for further biochemical analysis.

Table 1. Animal treatment protocol. The animals were weighed and randomly divided into 5 groups, (n=7). Naringin and distilled water were orally administered daily. BW = per kg body weight.

Treatment / Groups	**Distilled H_2O, (ml/BW) (Normal control)**	**Naringin, (mg/BW) (Normal)**	**Insulin, (U/ BW SC, b.d) (Diabetic)**	**Naringin, (mg/BW) (Diabetic)**	**Distilled H_2O, (ml/BW) (Control Diabetic)**
1	3.0	-	-	-	-
2	-	50.0	-	-	-
3	-	-	4.0	-	-
4	-	-	-	50.0	-
5	-	-	-	-	3.0

2.3 **Methods**

2.3.1 **Blood glucose testing**

FBG measurements were done on treatment days 0, 14 and 28, respectively. On treatment day 41, Glucose Tolerance Tests (GTT) were done after an overnight fast followed by a single intraperitoneal injection of 3.0 mg/kg BW of glucose in normal saline and blood glucose levels were measured at times 0, 15, 30, 60, 90 and 120 minutes. Insulin treatment for group 4 was withheld on the day of GTT. Blood glucose analysis was done as previously described and the calculated Area under the curve (AUC) from blood-glucose concentration-time curves presented as AUC units {(mmol/L) X Time (minutes)}.

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. The optical density was determined by a microplate reader (EZ Read 400, Biochrom®) at 450 nm.

Hepatic glycogen assay

Hepatic glycogen content was measured by the modified method of Seifter *et al.*,[~~24~~20]. Briefly, the liver tissue was homogenised in 1.0 ml of 30% KOH saturated with Na_2SO4. The homogenate obtained was dissolved by boiling in a water bath (100˚C) for 30 minutes, vortexed and cooled on ice. Glycogen was then precipitated with 2.0 ml of 95% ethanol, vortexed, incubated on ice for 30 min and later centrifuged at 550 g for 30 min. The glycogen pellets obtained were then re-dissolved in 1.0 ml of distilled water which was thereafter treated with 1.0 ml of 5% phenol and 5 ml of 96-98% sulphuric acid respectively. This was incubated on 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.

Determination of serum electrolyte and blood pH levels

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

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

$$AG = \{[Na^+] + [K^+]\} - \{[Cl^-] + [HC0^{3-}]\ [12 11]$$

Plasma thiobarbituric acid reactive substances (TBARS) assay

Plasma TBARS assay was carried out according to the modified method of Phulukdaree *et al* [25 21]**).** Briefly, 200 µl of plasma samples were added to 500 µl of 2% phosphoric acid (H_3PO_4), 400 µl of 7% H_3PO_4 and 400 µl of BHT/TBA solution in a set of clean glass test-tubes. In another set of eight clean fresh test tubes, 200 µl of serially diluted malondialdehyde (MDA) standard was added to 500 µl of 2% H_3PO_4, 400 µl of 7% H_3PO_4 and 400 µl of butylated hydroxytoluene (BHT)/ Thiobarbituric acid (TBA) solution. The reactions in both sets of tubes were initiated with 200 µl of 1.0 M HCl. All tubes were incubated in a shaking boiling water bath (100^{o}C) for 15 minutes and cooled at room temperature. n-butanol (1.5 ml) was then added to each tube and mixed thoroughly. Top phase (200 µl) was then transferred to a 96-well micro-plate in and read at 532 and 600 nm using micro-plate reader (Spectrostar®). Plasma MDA concentrations were calculated using an extinction coefficient of $1.56x10^5 M^{-1}cm^{-1}$.

Analysis of serum ketone body concentrations

Serum and urine ketone body levels were determined by the spectrophotometric enzymatic assay kit (Enzychrom ™, BioAssay systems, EKBD-100) according to the manufacturer's

instructions. The optical density (OD) was read at 340 nm using a spectrophotometer (Genesys 20, Thermospectronic) and the concentrations calculated according to the formula:

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

A similar procedure was repeated for the 3-β hydroxybutyrate (3-HB) assay using the 3-HB buffer, reagent and standard, respectively and the concentrations calculated using the formula:

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

Statistics

The data was presented as mean ± SD and analyzed by GraphPad Prism Software® Version 5.0. One-Way ANOVA or Student t-tests and non-parametric Mann–Whitney tests were used where applicable to determine statistical significance. Values of $P < 0.05$ were taken to imply statistical significance.

RESULTS

Animal growth change during treatment period

~~Diabetic~~ STZ only-treated groups exhibited significant ($p<0.0001$) weight loss compared to the normal control group. Treatment with insulin or naringin significantly ($p<0.0001$) increased weight gain compared to the non-treated ~~diabetic~~ STZ group. Treatment ~~of the normal non diabetic rats with~~ with naringin had no significant change in weight gain in normal rats compar~~ison~~ ed to the ~~normal~~ control (Figure 1).

Figure 1: Animal weight changes during treatment period. (***$p<0.0001$ compared to normal control, #$p<0.0001$ compared to ~~diabetic control~~non-treated STZ group~~.~~).

Water consumption during treatment period

The average daily water consumption was significantly (p<0.0001) higher in untreated ~~diabetic~~ rats compared to normal control group. However, water intake was significantly (p<0.0001) reduced in the insulin or naringin treated groups compared to the non-treated ~~diabetic~~STZ (Figure 2).

Figure 2: Water consumption during treatment period. (***p<0.0001 compared to normal control, #p<0.0001 compared to ~~diabetic~~ untreated STZ group~~control.~~).

Urine output

The urine output was significantly (p<0.0001) elevated in the untreated ~~diabetic~~ STZ~~rat~~ group in comparison to the normal control group. Treatment ~~of the diabetic group~~ with insulin significantly (p<0.05) reduced urine output in STZ treated animals compared to the ~~diabetic~~ untreated STZ~~control~~ group. However, naringin treatment did not have any significant effect on urine output in ~~diabetic~~ STZ-treated animals compared to the untreated ~~diabetic~~ STZ group~~rats~~ (Figure 3).

Figure 3: 24-hour urine output collected after the animals were put in metabolic cages. (***p<0.0001 compared to normal control, # p<0.05 compared to ~~diabetic control~~untreated STZ group~~.~~).

Glucose tolerance

~~Fasting blood glucose (~~FBG~~)~~ levels were significantly higher (p<0.0001) in the ~~diabetic~~ untreated STZ group compared to the normal control group. However, insulin but not naringin treatment significantly (p<0.05) improved the FBG levels compared to the ~~diabetic~~ untreated STZ group (Fig 4).

Figure 4: Fasting blood glucose levels measured by tail pricking after an overnight fast. (***p<0.001 compared to the normal control group, @#p<0.05 compared to ~~diabetic control~~untreated STZ group~~.~~).

The ~~diabetic~~ untreated STZ group~~s~~ showed glucose intolerance compared to the normal control (Fig 5A & B) with significantly (p<0.0001) increased~~. C~~ calculated ~~area~~ Area ~~under~~ Under the ~~curve~~ Curve (AUC) further ~~showed that the AUCs of the diabetic groups were significantly (p<0.0001) increased~~ compared to normal control group. N~~-but n~~either naringin nor insulin treatment significantly improved the AUCs in ~~diabetic~~ STZ-treated group~~s~~ (Figure 5C).

Figure 5: Glucose Tolerance Tests (GTT) after the animals were fasted overnight and challenged with intreperitoneal injection of 3.0 g/kg BW of glucose in normal saline in ~~non-diabetic~~normal animals treated with distilled water (controls) or naringin (**A**) and ~~diabetic~~ STZ-(**B**). Insulin treatment was withheld on the day of GTT and the figures were plotted in different graphs for clarity considering the large differences in blood glucose concentrations between normal and ~~diabetic~~ STZ groups on the y-axis. **C**; Calculated AUC from GTT. (***p<0.0001 to the normal control group).

Fasting plasma insulin

FPI concentration w~~as~~ere significantly (p<0.0001) lower in the ~~diabetic control~~STZ-treated group compared to the normal control group. ~~Treatment of diabetic groups with either n~~Naringin or insulin significantly (P<0.01) improved FPI concentrations in the STZ-treated compared to the untreated ~~diabetic~~ STZ ~~control~~group, respectively (Figure 6). Surprisingly, naringin treatment significantly increased FPI concentrations in ~~non-diabetic~~normal rats compared to the controls.

Figure 6: Plasma insulin concentrations after blood was collected by cardiac puncture. (***p<0.0001, ^p<0.05 compared to normal control, #p<0.05 compared to ~~diabetic control~~STZ animals).

Hepatic glycogen levels

Hepatic glycogen concentrations were significantly (p<0.0001) reduced in ~~the diabetic~~STZ-treated ~~group~~ compared to the control group. Treatment with insulin or naringin significantly (p<0.05) increased hepatic glycogen content compared to the ~~untreated diabetic control~~STZ only-treated group, respectively. Naringin treatment of normal rats caused significant (p<0.05) elevation of hepatic glycogen content compared to the normal control (Figure 7).

Figure 7: Glycogen levels in homogenised hepatic tissue. (***p< 0.0001, ^p< 0.05 compared to normal control, #p~~< 0.01, @ p~~<0.05 compared to ~~untreated diabetic~~STZ only-treated rats, respectively)

Plasma MDA concentrations in ~~diabetic control~~STZ only-trated group were significantly (p<0.0001) elevated compared to the normal control group. Naringin or insulin significantly (p<0.0001) reduced plasma MDA concentrations in ~~diabetic~~ STZ-trated rats compared to the ~~diabetic control~~STZ only treated group, respectively (Figure 8).

Figure 8: Plasma concentrations of ~~Malondialdehyde~~ MDA measured as a marker for lipid peroxidation. (***p<0.001 compared to normal control, #p<0.0001 compared to ~~untreated diabetic~~STZ only-teated group).

Serum β-hydroxybutyrate (3-HB) levels were significantly ($p<0.001$) elevated in the ~~diabetic~~ treated compared to the normal control group. Treatment with insulin or naringin significantly ($p<0.05$) reduced the levels of 3-HB compared to the ~~untreated diabetic~~STZ only-treated group, (Figure 9A).

Serum acetoacetate (AcAc) levels

Serum acetoacetate (AcAc) levels were significantly ($p<0.0001$) elevated in the ~~diabetic control~~STZ only-teated compared to normal control group. However, naringin significantly ($p<0.05$) reduced plasma AcAc levels in ~~diabetic~~ STZ-treated ~~rats~~ compared to the ~~diabetic control~~STZ only-treated rats (Figure 9B).

Figure 9: **A**; Serum 3-β-hydroxybutyrate (3HB) levels. (***$p<0.0001$ compared to normal untreated control, #$p<0.01$ and @$p<0.05$ compared to ~~untreated diabetic~~STZ only-treated rats) acetoacetate (AcAc) levels. (***$p<0.0001$ compared to normal ~~untreated~~ control, #$p<0.05$ ~~untreated diabetic~~STZ only-treated group)

Serum sodium, chloride and bicarbonate and blood pH were significantly ($p<0.01$, $p<0.0001$, $p<0.0001$ and $p<0.05$, respectively) reduced, while potassium was significantly ($p<0.05$) elevated in the ~~diabetic~~ STZ only-treated compared to the normal control group. ~~Treatment of the diabetic groups with i~~Insulin significantly ($p<0.05$) increased sodium and reduced potassium in the STZ-treated compared to ~~the untreated diabetic contro~~the STZ only-treated~~!~~ group, respectively. Naringin or insulin ~~treatment of diabetic rats~~ significantly ($p<0.05$) increased plasma bicarbonate and blood pH in STZ-treated compared to the ~~non~~STZ only-treated diabetic rats, respectively (Table II).

Table 2. Serum electrolytes and blood pH. (***p<0.0001, **p<0.01 and *p<0.05 compared to the normal control group, #p<0.0001, @p<0.01 and &p<0.05 compared to ~~untreated diabetic~~the STZ only-treated group).

	Normal control	**Normal naringin**	**~~Diabetic~~ STZ-insulin**	**~~Diabetic~~ STZ-N~~n~~aringin**	**~~Diabetic control~~STZ**
Sodium (mM)	142.70±0.56	142.80±0.37	140.00±1.00**&**	132.20±1.50	135.00±1.79****
Potassium (mM)	6.42±0.30	7.120±0.59	5.87±0.12**&**	7.34±0.59	7.56±0.46*
Chloride (mM)	103.80±0.89	103.00±0.63	100.30±1.33	96.20±2.40	96.40±1.36***
Bicarbonate (mM)	24.77±0.76	24.74±1.04	21.18±0.69 #	17.63±0.64 **@**	13.10±0.54 ***
Blood pH	7.4±0.24	7.33±0.20	7.33±0.17**&**	7.4.00±0.31**@**	6.64±0.30**

Anion gap (AG)

Calculated AG was significantly (p<0.0001) increased in the ~~diabetic~~ STZ only-treated group compared to the normal control group. Naringin or insulin treatment significantly (p<0.05) decreased the serum AG in the ~~diabetic~~ STZ-treated groups compared to the ~~untreated diabetic~~STZ only-treated group, respectively (Figure 10).

Figure 10: Anion gap (AG) calculated from serum electrolytes as previously described [~~12~~11]. (#p< 0.0001 compared to normal control group, *p< 0.05 compared to the ~~untreated diabetic~~STZ only-treated group).

DISCUSSION

This study was based on a typical type 1 diabetes model created with STZ which selectively and irreversibly completely destroys the pancreatic β-cells by oxidative damage. Interestingly, ~~treatment of both normal and diabetic rats with~~ naringin treatment of normal rats was associated with relative increase in FPI concentrations (Fig 6) ~~yet naringin is not known to be insulinotropic~~. Considering that insulin treatment similarly increased FPI in diabetic animals, we speculate that the antioxidant effects of naringin reduced oxidative stress in the pancreatic β-cell mitochondria leading to increased ATP synthesis and subsequent insulin secretion. By its anabolic effects, insulin similarly to naringin, boosted residual anti-oxidant capacity of the β-cells leading to increased insulin secretion. This could have limited oxidative damage of STZ to allow the β-cells to retain some residual activity. Potential insulinotropic effects of naringin have only recently been demonstrated [22].

~~Diabetic~~ STZ-treated rats exhibited significant weight loss compared to controls (Fig 1). It may be argued that diabetes conferred hypophagic state since we did not measure food intake but it should also be considered that loss of body mass is a well known phenomenon in type 1 diabetes irrespective of food consumption rates [3, ~~17~~13, ~~19~~15]. That naringin, like insulin significantly attenuated weight loss in diabetic rats further suggests that, weight loss in diabetic rats was due to lipid and protein catabolism (associated with insulin deficiency) which ~~was~~ were inhibited by both naringin and insulin, respectively. Hence we speculate that antioxidant effects of naringin reduced oxidative stress and free radicals which are known to provoke the release of catabolic insulin counter-regulatory hormones [~~10~~9].

Polydipsia and polyuria were also observed in ~~diabetic~~ STZ-treated animals compared to the normal control group (Fig 2 and 3). Hyperglycemia in DKA leads to increased extracellular osmolality which ~~draws fluid from the intracellular compartment which is then lost through renal diuresis causing~~causes dehydration. As a compensatory mechanism, increased osmolality activates hypothalamic osmoreceptors causing the release of antidiuretic hormone (ADH)

which corrects the hyperosmolar state [~~26~~23]). Failure of this mechanism leads to activation of thirst leading to the increased water intake. Insulin ~~treatment of the diabetic rats~~ reversed both polyuria and polydipsia in STZ-treated rats. However, naringin treatment reversed only polydipsia but not polyuria suggesting that naringin inhibited thirst activation but had no anti-diuretic effects either in the normal or ~~diabetic~~ STZ-treated rats.

Unlike insulin, ~~treatment of the diabetic rats with~~ naringin improved neither FBG nor glucose intolerance in ~~diabetic~~ STZ-treated rats (Fig 4). We previously reported that naringin does not ameliorate hyperglycemia in diabetic type 1 rats [~~17~~13]). This is contrary to other studies that have reported hypoglycemic effects of naringin and its aglycone naringenin to be mediated by suppression of hepatic expression of key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [~~17~~13-~~19~~15, ~~21~~17, ~~27~~24]). We concur with these observations but opine that these studies were done in simulated type 2 diabetes models where there was insulin resistance but not absolute deficiency. That insulin treatment significantly improved fasting blood glucose and plasma insulin, respectively suggests that by its anabolic effects insulin could have promoted modest pancreatic β-cell recovery in STZ- treated animals, enhanced residual insulin secretory capacity or both (Fig 4, 5B and 6). Glucose tolerance was similar between naringin-treated and control rats but calculated AUC suggested that ~~diabetic~~ STZ-treated rats were significantly glucose intolerant compared to normal controls (Fig 5). GTT was conducted with intraperitoneal injections of 3.0 mg/kg body weight of glucose in normal saline and even though we could not measure concurrent plasma insulin response, our results suggest that ~~diabetic~~ STz-treated rats were already in hyperosmolar state considering the high fasting blood glucose concentrations (Fig 4 and 5 B). Since insulin treatment was withheld during GTT, it is therefore not surprising that glucose tolerance did not improve in the insulin-treated group compared to ~~non-treated diabetic~~STZ only-treated group.

Liver glycogen content was significantly reduced in ~~non-treated diabetic~~STZ only-treated rats controls but this was significantly reversed by either naringin or insulin treatment ~~of diabetic~~ suggests that in the absence of insulin, glucagon and other insulin counter-regulatory hormones promoted gluconeogenesis and glycogenolysis which reduced glycogen storage in the liver in ~~diabetic~~STZ-treated rats but this was inhibited by either naringin or insulin treatment. Naringin treatment of normal rats interestingly significantly increased hepatic glycogen content compared the normal controls (Fig 7). Insulin is known to activate glycogen storage by increasing expression of glycogen synthase which catalyses the rate-limiting step in glycogen synthesis [~~28~~25]. Even though our study did not investigate the direct effects of naringin on hepatic expression of glycogen synthase, previous studies have shown that naringin or its aglycone naringenin suppresses PEPCK and G6Pase activities via activation of AMP-activated protein kinase (AMPK) [(~~29~~26, ~~30~~27]. It is therefore likely that in our study, naringin failed to hypoglyecemic effects due to insulin deficiency and hence argue that naringin has metformin-like effects.

~~Hyperglycemia-associated o~~Oxidative stress causes lipid peroxidation due to increased Reactive Oxygen Species (ROS) leading to overproduction of MDA, which is used as a biomarker [~~31~~28, ~~32~~29]. Lipid peroxidation was significantly elevated in the ~~diabetic~~ STZ-treated compared to the control group but was significantly reversed by naringin treatment (Figure 8). Oxidative stress stimulates production of pro-inflammatory cytokines which ~~provoke~~ cause the release of insulin counter-regulatory hormones that are known to ~~provoke~~ promote ketoacidosis [3]. Therefore, the antioxidant effects of naringin either directly or through enhancing antioxidant defence systems directly or by augmenting insulin secretion reversed ketoacidosis. This is demonstrated by the significantly reduced plasma 3-HB and AcAc in diabetic rats that were treated with naringin compared to the untreated diabetic group (Fig 9 A and B). Insulin significantly reduced plasma 3-HB compared to untreated diabetic

animals suggesting that naringin like insulin could be having a direct inhibitory effect on hydroxymethyl glutayryl Coenzyme A (HMGCoA) synthase, which catalyses the rate-limiting step in ketogenesis [~~10~~9].

Jung et al., [~~28~~25] previously showed that naringin suppresses plasma carnitine palmitoyl-O-transferase (CPT) which is involved in the transport of free fatty acids (FFA) across the mitochondrial membrane for β-oxidation and ketogenesis. Therefore, with reduced CPT levels, there is reduced ketogenesis due to decreased supply of FFA. Increased ketogenesis observed in diabetes is associated with overproduction and accumulation of ketone bodies which are strong organic anions. These dissociate freely producing hydrogen ions which bind and overwhelm serum bicarbonate buffering capacity eventually leading to metabolic acidosis in diabetes. However, ~~treatment of the diabetic rats with~~ naringin significantly improved plasma bicarbonate and blood pH levels, ~~(Table 2)~~ compared to ~~untreated diabetic~~STZ only-treated group (Table 2). Subsequently acid-base imbalance marked by increased AG was significantly reversed by naringin ~~treatment of diabetic~~ rats compared to ~~the non~~STZ only-treated ~~ones~~ group (Fig 10). However, unlike insulin, naringin did not correct electrolyte imbalance in diabetic animals suggesting that like with insulin treatment, fluid replacement therapy should precede naringin supplementation were it to be used clinically. These observations confirm that naringin reversed metabolic acidosis associated with DKA albeit in experimental animal model. We recently reported antidiabetic effects of grapefruit juice in experimental animal studies [~~33~~30]. Naringin is the main flavanoid in grapefruit juice and therefore it seems plausible to infer that some of the antidiabetic effects of grapefruit juice could be attributed to naringin in addition to other as yet unidentified bioactive chemical ingredients. However, naringin on its own appears in this study to be associated with favourable metabolic end-points in type 1 diabetes. There is no known toxicity of naringin both to humans or experimental laboratory animals [~~34~~31, ~~35~~32] and we used pharmacologically effective 50 mg/kg body of

naringin [~~17~~13, ~~36~~33, ~~37~~34] owing to its poor solubility in water which was dose limiting by gavage. Considering the failure of known endogenous antioxidants like vitamins E and C to exert positive pharmacological effects on degenerative metabolic diseases in large scale clinical trials [~~35~~32], our results presented here suggest that citrus-fruit-derived flavanoids like naringin could be viable cheaper alternatives.

Conclusion.

Although naringin did not improve glucose intolerance, it was associated with reversal of weight loss, improved glycogen storage and insulin secretion in diabetic rats. However, naringin abrogated metabolic acidosis suggesting a role in the management of DKA. These actions of naringin appear to be mediated in part by its powerful antioxidant affects. Our findings therefore suggest that naringin as a nutritional supplement could be protective against ketonemia in type 1 diabetes patients and may be used as adjunct therapy pending further clinical studies.

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5. Review Comments to the Author

Please use the space provided to explain your answers to the questions above. You may also include additional comments for the author, including concerns about dual publication, research ethics, or publication ethics. (Please upload your review as an attachment if it exceeds 20,000 characters)

Reviewer #1: The present study reported Narigin may improve the oxidative stress and ketoacidosis but have no effect on glucose tolerance in type 1 diabetic rat, which is interesting and challenging. There are still some concerns need to be addressed.

1. In the current study, hypoglycemic effect of Naringin was not observed which was inconsistent with other reports. More discusses are needed to interpret the increased insulin and hepatic glycogen but no influence on glucose by Naringin treatment. Would the potential hypoglycemic effect of Naringin exert on mild condition? Mild diabetic rats are suggested to use as an additional group (e.g.STZ 35 mg/kg). Have the authors try to confirm its insulinotropic effect on cell lines? Or other pathways for example, stimulating the secretion of incretin when oral GTT performed? (J endocrinology, 2013,217:185-196)

Response:

As highlighted in our discussion, hypoglycemic effects of naringin so far demonstrated are apparent in type 2 diabetes rat models where there is some residual insulin secretion. We have previously shown that without insulin, naringin does not exert hypoglycemic effects {J Cardiovasc Pharmacol. 2012 Feb;59(2):133-41; Accepted Manuscript in PLOS ONE (PONE-D-15-28866R2)}. Enhanced insulin secretion in normal non-diabetic rats treated with insulin could be attributed to antioxidant potential of naringin that reduces reactive oxygen species concentrations in the pancreatic β-cells leading to increased ATP production and insulin secretion (our as yet unpublished work on the effects of naringin on insulin secretion in RIN-5F cell lines). Insulinotropic effects of naringin have not previously been fully investigated but is a possibility. DPP-IV inhibitory potential of naringin has recently been described (Diabetes Res Clin Pract. 2012 Jul;97(1):105-11) suggesting that naringin could have increased insulin secretion in these rats by increased GLP-1 half-life. However, enhanced insulin secretion was an unexpected finding that was beyond the scope of our study in the interim.

Similarly, we unexpectedly found that naringin significantly increased glycogen content in non-diabetic rats compared to controls. However, this is not surprising considering our recent finding (Eur J Nutr. 2016 Mar;55(2):631-638) that grapefruit juice significantly increases hepatic glycogen content in non-diabetic and diabetic compared to controls and diabetic rats, respectively by activating glucokinase and down-regulating hepatic activities of key gluconeogenic enzymes, PEPCK and G-6-Pase. Interestingly, insulin treatment of diabetic rats did not significantly increase glycogen concentrations compared to untreated diabetic rats. Naringin is the predominant bioflavonoid in the grapefruit juice and we are convinced that in the previous and present study, it activated

glycogen synthase via AMPK upregulation like metformin (Arch Biochem Biophys. 2012 Feb 1;518(1):61-70). These points have been incorporated into the discussion in the present manuscript.

2. STZ treatment may take some days to exert effects stably (generally 5-7 days). Why the authors confirm the blood glucose on day 3 after STZ treatment? Similarly, treatments were performed immediately following the confirmation. STZ induced diabetic model may lead to wide variations in weight and FBG. Some rats may display very severe symptom (e.g. FBG > 18 mmol/L, weight <180 g) following the STZ treatment. How did authors deal with this? Details of grouping are needed.

Response:

We have been consulted by other research groups on how we manage to induce stable hyperglycemia within 24 hrs of administering STZ without animal deaths. In our experience, the animals have to be fasted overnight, and STZ dissolved in 0.2 ml of 0.1 M citrate buffer, pH 4.5. It is critical that be buffer be made a fresh and the pH kept at 4.5 without adjusting with either NaOH or NaCl. In this way blood glucose concentrations remain stable and no adverse effects have been experienced in our studies

3. For the weight, water intake and urine output changes showed, it is better to provide a weekly line chart which may reflect the details and fluctuations.

Response:

We have previously tried that but the figures become cluttered due to large number of animals and the critical information would be blurred. Fluctuations in weights, water consumptions and urine outputs do occur during treatment in response to such situations as overnight starvation but the overall terminal changes are more critical in conveying the intended message than piecemeal effects of interventions.

4. Please unify the markers used for indicating significance which were disordered and confusing (&@*#^). For instance, may use # for comparing with normal control; * for untreated diabetic group;

Response:

Done

5. Abbreviations should not be used when the item firstly appears. Line 39, FPI; Line 138, FBG.

Response:

Done

Reviewer #2: The authors report the effects of 40-day daily treatment with the main flavonoid of grapefruit, Naringin, to a cohort of SZT-injected rats as a model of Type-1 Diabetes. Control cohorts included SZT-treated and normal rats, given either water or naringing, as well as a reference cohort of SZT-treated rats given insulin twice daily. After the 40-day treatment the cohorts were subjected to IPGTT using 3.0 g/kg dextrose by intraperitoneal

injection. The next day the animals were sacrificed and blood was collected for a number of measurements, including insulin, ions, and metabolites. The authors describe the differences in ketoacidosis biomarkers between the Naringing-treated cohorts and control rats, suggesting that this treatment may improve ketoacidosis, which is the main message of this manuscript. The molecular target and physiological mechanism is not understood, and this cannot be known from exploratory in vivo experiments. However, the authors go to great lengths to hypothesize, but this seems unnecessary and potentially the most deterring aspect of the lengthy discussion section. The authors report and underscore a lack of significant differences between Naringin-treated and control rats in the IPGTT experiment. However, because of the relatively large dose of sugar used (in particular for a T1D model) this single GTT experiment is insufficient to fully support the claim of the authors. One further recommendation is changing the manuscript title to represent only the positive observations, for example: "Grapefruit compound Naringin improves ketoacidosis metabolites and lipid oxidation and in Type-1 Diabetes rat model". Editorial recommendation: major revisions.

Response:

We have previously reported inability of naringin to improve glucose intolerance in rats treated with STZ after a similar glucose challenge (J Cardiovasc Pharmacol. 2012 Feb;59(2):133-41) hence our observation here is not isolated. Naringin has not be demonstrated to be an insulin secretagogue and its proposed insulinotropic effects are apparent only in simulated type 2 diabetes models (Diabetes Res Clin Pract. 2012 Jul;97(1):105-11). Currently available scientific evidence suggests that naringin and its aglycone naringenin could be acting like insulin sensitizers just like metformin (Arch Biochem Biophys. 2012 Feb 1;518(1):61-70; Biochem Biophys Res Commun. 2010 Jul 23;398(2):178-83) by activating AMPK. In this context, it is not surprising that we did not observe improved glucose tolerance in a type 1 diabetes rat model. It cannot be argued that 3.0 g/kg body weight glucose used in this study was too high to show differences in IPGTT since even in non-STZ-treated rats, there were no significant difference in glucose tolerance between naringin-treated and control rats. A lower dose of glucose could have produced a flat curve considering the difference in blood glucose concentrations between STZ-treated and non-STZ-treated rats.

With anticipated permission of the Editor, we have changed the title accordingly

Main comments:

1) The introduction and discussion are too long and particularly focused on ketoacidosis as the main source of all ailments in diabetes. Major revision is required to remove sensationalistic language, please use ONLY standard scientific language that describes facts and credit associated literature references. To be very specific, replace the words/sentences: "courtesy", "exacerbate", "catabolic nature", "contentious issues", "random blood glucose", "diabetic state"; as well as convoluted/incorrect concepts like "pro-hyperglycemia insulin counter-regulatory hormones", "freely producing hydrogen ions which bind an overwhelm serum bicarbonate buffering capacity eventually leading to metabolic acidosis", "hyperglycemia-associated oxidative stress causes lipid peroxidation due to increased ROS leading to

overproduction of MDA". There are also a number of unsupported statements like "offer some hope", "devoid of adverse effects", "abrogated metabolic acidosis", "metformin-like effects" and "demonstrated powerful antioxidant effect". Note that "powerful" is not an objective and meaningful word in this context (i.e. low redox potential of the molecule? binds with high affinity to a target?).

Response:

Noted. The offensive words/phrases have been purged where appropriate and the introduction and discussion sections shortened. However, we need to point out that the study was designed primarily to investigate the effects of naringin on diabetic ketoacidosis with glucose intolerance as a confirmation of diabetes and oxidative stress as a possible mechanism of action. Diabetic ketoacidosis is not a source of diabetes but rather a metabolic complication besides nephropathy, cardiomyopathy, retinopathy and neuropathy which we were not intent on investigating in this setting.

We see no problem with using some of the phrases such as "random blood glucose" which is clinically applicable as opposed to "fasting blood glucose". Similarly we see nothing wrong with referring to naringin as having 'metformin-like effects" in the control of blood glucose as it appears to be an emerging consensus among different investigators in this field.

2) All instances of the sentence "Diabetic groups" (or "diabetic rats") should be changed to "SZT-treated group". Similarly, "untreated diabetic group" would be substituted with "SZT-treated control cohort", etc.

Response:

Done

3) Figure 1 shows the BW at the end of the study, please show a longitudinal scatterplot. Figure 4 shows only the fasting glucose on day 28 of treatment, however, day 0 and 14 were also measured. This data should be included.

Response:

Done.

4) Fasting plasma insulin (FPI) was measured on day 42, which is 1-day post GTT on day 41. Therefore, this protocol must have required 2 nights of fasting in a row with a short ad libitium feeding time in between, which is not a standard recovery post-GTT. This needs to be clarified and stated because it may possibly lead to differences with other study designs in the future.

Response:

Fasting blood glucose were done on days 0, 14 28 and 41 but not 42 as part of IPGTT. The error has been corrected. The animals were then sacrificed on day 42.

4) The formulas for concentrations of [AcAc] and [3HB] are not consistent with concentration units. Please provide literature references for these methods. Please provide a reference for the quoted extinction coefficient of 1.56x10^5 for the MDA

assay. Similarly, it would seem that the measurement of glycogen would require a standard curve to convert the spectrophotometer measurements to mg/g liver protein. Please include all data, literature references, and methods needed.

Response:

Anion Gap (AG) was calculated according to: Clin J Am Soc Nephrol. 2007 Jan;2(1):162-74, (reference no 11 in the revised manuscript) and expressed as mM (mmol/L) which is also the same as mEq/L. References for MDA (S Afr J Sci 2010;106:1-5) and glycogen determination (Arch Biochem 1950;25(1):191-200) are provided in the methods sections in the manuscript. Standard curves were used to determine the concentrations of the unknowns in all experiments and would not add value to the information on the figures unless provided as supplementary data.

5) The single IPGTT experiment using a 3 g/kg dose of sugar is inconclusive because the resulting measurements ~30 mmol/L fall outside the "dynamic range" of blood glucose homeostasis. As observed in Figure 5B, these animals lacking beta-cells cannot effectively respond to such doses of sugar, and do not return to baseline even after 2.5 hr. Therefore, lack of differences in this experiment are insufficient evidence to support the negative claim. The manuscript should be modified to take into account this possibility.

Response:

Whatever the amount of glucose administered, blood glucose concentrations never return to baselines in GTT even after 2 hrs in diabetic individuals and this is one of the criteria clinically used to diagnose diabetes. Fasting blood glucose concentrations in STZ-treated rats were above 25 mM before loading glucose anyway, meaning that any amount of glucose loaded would have spiked blood glucose levels beyond physiological range. In non-STZ-treated rats, 3.0 g/kg did not increase blood glucose concentrations beyond physiological ranges and there were no differences between controls and naringin-treated groups even in the presence of insulin suggesting lack of hypoglycemic effects of naringin in normal or low insulin concentrations. A lower dose of glucose could have produced a flat curves considering the differences in blood glucose concentrations between STZ-treated and non-STZ-treated rats. Hypoglycemic effects of naringin are reported in simulated type 2 diabetes models where there is hyperinsulinemia (Nutrients. 2013 Feb 27;5(3):637-50).
