ANTI-DIABETIC AND ANTI-DYSLIPIDAEMIC EFFECTS OF GRAPEFRUIT JUICE

SANELISIWE PRECIOUS NGUBANE

UNIVERSITY OF KWAZULU-NATAL

ANTI-DIABETIC AND ANTI-DYSLIPIDAEMIC EFFECTS OF GRAPEFRUIT JUICE

SANELISIWE PRECIOUS NGUBANE

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ANTI-DIABETIC AND ANTI-DYSLIPIDAEMIC EFFECTS OF GRAPEFRUIT JUICE

BY

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A Dissertation submitted in partial fulfilment of the requirements for the award of the degree Master of Medical Science (Pharmacology), in the Department of Pharmacology, Discipline of Pharmaceutical Sciences, Faculty of Health Sciences, University of KwaZulu-Natal, Durban 4000, South Africa

Supervisor**: Dr PMO Owira**

Durban

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As the candidate‘s supervisor, I have approved this thesis/dissertation for submission.

Signed: Name: Date:

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DECLARATION

I hereby declare that the work presented in this thesis is original and that all ideas and opinions expressed in this article are my own creation. This work has not been presented in part or in full by me to any other university for any other degree. Materials that have been referenced have been acknowledged in full in the text. There were no _ompeting interests'.

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Dr PMO Owira

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Supervisor

2015

DEDICATION

This work is dedicated to my mother and sister, Buyisiwe Ngubane and sister, Sthembile Ngubane, for standing with me and believing in me even when I least believe in myself. For supporting my dreams and aspirations even when they may have made little sense; this one is for you!

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Finally and most importantly, to my Lord, Saviour and Father; like Paul –I count all my achievements loss in comparison to knowing you". Thank you Abba!

ABBREVIATIONS

ABBREVIATION	DEFINITION
α	Alpha
α-Glycerol-P	Alpha-Glycerol-Phosphate
ACAT-2	Acetyl-Coenzyme A acetyltransferase 2
ACE-I	Angiotensin Converting Enzyme Inhibitor
ADA	American Diabetes Association
AGE	Advanced Glycation End-products
AIDS	Acquired Immunodeficiency Syndrome
ALE	Advanced Lipoxidation End-products
AlipoB	Alipoprotein-B
AMP	Adenosine Monophosphate
AMPK	Adenosine Monophosphate-Activated Protein Kinase
ANG II	Angiotensin II
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
β	Beta
BP	Blood Pressure
BW	Body Weight
Ca^{2+}	Calcium ions
cAMP	Cyclic Adenosine monophosphate-phosphodiesterase
CETP	Cholesterol Ester Transfer Protein
CNS	Central Nervous System

CTS	Carpal Tunnel Syndrome
CYP3A4	Cytochrome P450 3A4
D60	Diabetic, 60 mg/kg
DAG	1, 2~diacylglycerol
δ	Delta
DHA	Decosahexaenoic Acid
DHAP	Dihydroxyacetone Phosphate
DKA	Diabetic Ketoacidosis
DM	Diabetes Mellitus
DPP	Dipeptidyl Peptidase
EDTA	Ethylenediaminetetraacetic Acid
EPA	Eicosapentaenoic Acid
ESRD	End-of Stage Renal Disease
ESRF	End-of Stage Renal Failure
FBG	Fasting Blood Glucose
FFA	Free Fatty Acid
Fructose-6-P	Fructose-6-Phosphate
γ	Gamma
GAD	Glutamic Acid Decarboxylase
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFAT	Glutamine:Fructose-6-phosphate Amidotransferase
GFJ	Grapefruit Juice
GFJ-D60	Grapefruit juice and Diabetic, 60 mg/kg
GFJ-ND	Grapefruit juice and non-diabetic
GLP	Glucagon-Like Peptide

Glucosamine-6-P	Glucosamine-6-Phosphate
Glucose-6-P	Glucose-6-Phosphate
GLUT4	Glucose Transporter-4
Glyceraldehyde-3-P	Glyceraldehyde-3-Phosphate
GSH	Glutathione
G6Pase	Glucose-6-phosphatase
H_2SO_4	Sulfuric Acid
HbA_{1c}	Glycosylated haemoglobin
HBP	Hexosamine Biosynthesis Pathway
HDL	High Density Lipoprotein
HDL-C	High Density Lipoprotein Cholesterol
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HIV	Human Immunodeficiency Virus
HMG-CoAR	3-Hydroxy-3-Methyl-Glutaryl-CoA Reductase
IDDM	Insulin-Dependent Diabetes Mellitus
IDP	Inositol Diphosphate
Ins-D60	Insulin and Diabetic, 60 mg/kg;
K^+	Potassium
KCl	Potassium Chloride
Km	1/slope
KOH	Potassium Hydroxide
LADA	Latent Autoimmune Diabetes in Adults
LDL	Low Density Lipoprotein
LDL-C	Low Density Lipoprotein Cholesterol
LpL	Lipoprotein Lipase

$MgCl_2$	Magnesium Chloride
MODY	Maturity Onset Diabetes of the Young
Na^+	Sodium
Na_2CO_3	Sodium Carbonate
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide Hydride
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NIDDM	Non-Insulin-Dependent Diabetes Mellitus
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
OAA	Oxaloacetate
OATP	Organic Anion-Transporting Protein
OGTT	Oral Glucose Tolerance Test
OTC	Over-The Counter
PCOS	Polycystic Ovary Syndrome
PEPCK	Phosphoenolpyruvate carboxykinase
PKC	Protein Kinase C
PPAR	Peroxisome Proliferator-Activated Receptor
RAPD	Random Amplified Polymorphic DNA
RAS	Renin-Angiotensin System
RBT	Random plasma (Blood) glucose Test
ROS	Reactive Oxygen Species
SCAR	Sequence Characterized Amplified Region
SDS	Sodium Dodecyl Sulphate

SDS–PAGE	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis
STZ	Streptozotocin
SUR	Sulfonylurea Receptors
TB	Tuberculosis
TG	Triglycerides
TZD	Thiazolidinedione
UDP	Uridine Diphosphate
UDP-GlcNAc	Uridine Diphosphate- *N*-acetyl glucosamine
UNE	Ulnar Neuropathy at the Elbow
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoprotein
V_{max}	Maximum Velocity
TC	Total Cholesterol

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Glucokinase activity was plotted against substrate concentrations.

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ABSTRACT

Background

Hypoglycaemic effects of grapefruit juice in diabetic rats have been previously reported. The mechanisms by which grapefruit juice lowers blood glucose are not known. This study aimed to investigate the hypoglycaemic and anti-dyslipidaemic effects of grapefruit juice, as well as to elucidate the possible mechanism/s of action of this juice.

Materials and Methods

Male Wistar Rats (*Rattusnovergicus*) of 200-300 g body weight (BW) were randomly divided into five groups (n=6). Animals in group 1 were treated with 3.0 ml/kg of water for 60 days, by oral gavage. Groups 2, 4 and 5 were rendered diabetic by a single intraperitoneal injection of 60 mg/kg BW of streptozotocin. Group 5 was further treated with 4.0 U/kg of insulin (subcutaneously, twice daily), while groups 3 and 4 were orally treated with 3.0 ml/kg of GFJ.

Fasting blood glucose and glucose tolerance tests were done in all the groups. Plasma insulin levels were also measured. Hepatic glycogen content, glucokinase and glucose-6-phosphatase (G6Pase) activities were measured in homogenised liver tissues. Plasma lipid levels were measured and hepatic enzymes (Acetyl-Coenzyme Aacetyl Transferase (ACAT) and 3-Hydroxy-3-Methyl-Glutaryl-CoA (HMG-CoA reductase) expression was determined.

Results

Diabetic rats showed significantly reduced weight gain, but higher water consumption in comparison to the controls. Fasting blood glucose was significantly higher in the diabetic group compared to controls, but were significantly ($p<0.05$) attenuated in GFJ-treated diabetic group, compared to the control. Diabetic rats exhibited significantly impaired

glucose tolerance compared to controls, which was, however, improved in GFJ-treated groups in comparison to the diabetic non-treated group. GFJ treatment did not improve fasting plasma insulin in diabetic animals. Glucokinase activity and hepatic glycogen concentrations were significantly increased by GJF treatment, but G6Pase was alternatively suppressed by GFJ treatment. HDL-C levels were significantly increased in GFJ treated diabetic animals. Liver ACAT and HMG-CoA reductase enzyme expression were significantly suppressed in GFJ treated diabetic animals in comparison to the non-treated diabetic animals.

Conclusion

The findings show that GFJ has both hypoglycaemic and anti-dyslipidaemic effects. Although it not insulinotrophic, GFJ improves glucose intolerance in diabetic animals by supressing hepatic gluconeogenesis. Furthermore, GFJ improved plasma lipid profiles and supressed the liver expression of ACAT and HMG-CoA reductase enzymes.

Key words: grapefruit juice, diabetes mellitus, diabetic dyslipidaemia, insulin, lipids, ACAT, HMG-CoA reductase.

CHAPTER 1

Literature Review and Introduction

1.1 Epidemiology of diabetes mellitus

Diabetes Mellitus is a metabolic disorder with genetic, environmental or acquired causes [1-3], and is characterised by chronic hyperglycaemia as well as altered carbohydrate, protein and fat metabolism [4, 5]. These result from insulin resistance, the inability of the body to produce insulin, or the combination of both [6].Diabetes usually presents itself with a cocktail of symptoms such as excessive thirst, blurring of vision, polyuria and weight loss [4]. These symptoms are sometimes not severe, and may at times even be absent, but the hyperglycaemia present may be enough to cause both pathological and functional changes, which may be present when the diagnosis is made [4]. Diabetes is classified into four main classes namely Type1, previously known as Insulin-Dependent Diabetes Mellitus (IDDM), Type 2previously known as Non-Insulin-Dependent Diabetes Mellitus (NIDDM), gestational diabetes and other specific types [7]. Type 2 diabetes is the most prevalent diabetes worldwide [5, 7, 8].Other types of diabetes include a varied group of conditions which may be of genetic, surgical, malnutrition and infections origins, which may lead to symptoms and manifestations of diabetes [7]. Maturity Onset Diabetes of the Young (MODY), is classified under other specific types of diabetes [7, 9].

It was predicted that the prevalence of diabetes worldwide would reach 150 million people in the year 2000; and this number was expected to double to 300 million people by the year 2010 [8, 10]. By the year 2025 it is predicted that type 2 diabetes alone will be affecting more than 300 million people worldwide [11]. Increased longevity and urbanisation, lifestyle changes, obesity and dietary modifications are expected to increase the global incidences of

Type 2 diabetes [8, 9]. In the 1980‘s, there was very little epidemiological data on diabetes in the continent of Africa [12-15], with most data in the 1980‘s indicating diabetes prevalence of between 0% -1.4% in Africa [10, 14, 15]. The reports of the incidences of diabetes in the continent of Africa have in the past two decades changed, with diabetes presenting itself as a significant medical problem in the developing countries of Africa [10].

Diabetes in Africa

From the low diabetes prevalence of between 0% to 1% observed among Africans in the 1980‘s, the diabetes burden reached 20% in the 2000‘s, and is predicted to be affecting between 18.6 to 23.9 million Africans by the year 2030 [13, 14]. This would be a 98% rise from the 12.1 million seen in 2010 [13]. In African countries, diabetes has a higher prevalence in urban areas, where there is low physical activity, and where people indulge more in diets high in fat and sugars [9, 14]. Furthermore, obesity, one of the greatest contributing factors of diabetes [9], is very high Africa, with equal prevalence among all ethnic groups and age groups, but a higher prevalence among females than males [14]. There is also evidence of the racial predilection in the number of individuals suffering from diabetes [10, 13-15]. In most African countries, such as Kenya, Mali, Mozambique, Zambia and Nigeria; Africans of European descent are most affected by diabetes, followed by Indians and finally by Blacks. This racial predilection is attributed to genetic causes [14].

In South Africa, like most African countries, there is a rural-urban gradient that is observed in the prevalence of diabetes. In the year 2000, the South African National Burden Disease Study reported that diabetes was the tenth leading cause of morbidity and mortality in South Africa, accounting for about 2.6% of all deaths, which represents to 20000 deaths, (4.3%) of the South African population [16]. However, these estimates may be lower than current numbers considering time lapse.

The burden of diabetes in South Africa and Africa holistically is far too high, and improvements in treatment/management strategies as well as preventive measures that can delay the onset of diabetes should be employed [16]. One of the greatest challenges in the management of diabetes and other pandemics in Africa at large is the burden of these diseases, in comparison to the technology and the cost of managing these diseases [13, 14, 16]. Hence, more inexpensive yet effective strategies to reduce the burden of diabetes should be employed. Such strategies include, over and above current approaches, the use of plant and plant materials in the prevention, treatment and possibly cure of diabetes.

1.2 Pathogenesis of diabetes

1.2.1 Blood glucose homeostasis

The endocrine pancreas contains three different types of secretory cells which function in the secretion of glucagon (α-cells), insulin (β-cells) and somatostatin as well as gastrin (δ-cells) [17]. The secretion of insulin promotes the uptake of glucose, amino acids and fatty acids. It also increases glycogen synthesis and storage in the liver from glucose [17]. Somatostastin functions in the regulation of the release of insulin and glucagons, decreasing the motility of gastrointestinal tract and growth hormones secretion [17]. During the fed state, carbohydrates are broken down into glucose, fructose and galactose by gastrointestinal tract enzymes [17]. These sugars are then actively and passively absorbed, and transported by transporters from epithelial cells into capillaries. Once the glucose reaches the blood stream, it is taken up into pancreatic cells. The increased concentration of glucose inside pancreatic cells stimulates the release of insulin, which then diffuses into the portal vein, where it is transported by the blood into the liver at the same time with digested food [5, 17]. Insulin thus secreted acts on tissues that store energy, such as the liver, skeletal and adipose tissues [5]. During fasting states, the glucose concentration is decreased, pancreatic α-cells release glucagon, and

pancreatic β-cells reduce the amount of insulin secreted [5]. Glucagon then mobilizes glucose from the liver by increasing glycolysis and gluconeogenesis. When the individual continues with fasting, catecholamine and glucocorticoid concentrations increase, leading to the release of fatty acids; the break-down of protein and adipose tissues [17].

It is critical that plasma glucose concentrations be maintained, as it is an energy source for delicate organs like the Central Nervous System (CNS) [5]. The CNS cannot produce nor store glucose for long periods and the smallest levels of hypoglycaemia can cause drastic CNS dysfunctions. To prevent for CNS dysfunction as a result of hypoglycaemia, glucose regulatory systems have evolved to reduce hypoglycaemia. Under normal conditions, plasma glucose concentrations are maintained at a narrow range of between 3 and 5.5 mmol/L [5, 7]. This range is maintained by insulin that is secreted from beta cells of the pancreas [7], in spite of variations in glucose levels that occur after meals and exercise [5]. Fasting blood glucose levelsabove6 mmol/L are associated with the progression of diabetes [7].

1.2.2 Diagnosis of diabetes mellitus

Diabetes Mellitus is diagnosed using one or a combination of the following methods, which primarily measure glycaemia [9]. The three major tests used to measure blood glucose levels include the Random Blood glucose Test (RBT), Fasting Blood glucose Test (FBG), as well as the two hour post glucose load Oral Glucose Tolerance Test (OGTT) [5, 7, 9]. The presence of diabetes is diagnosed by glucose levels of greater than 11.0 mmol/L for RBT, in combination with symptoms of diabetes [5, 7, 9] and >7.0 mmol/L for the FBG, according to the American Diabetes Association (ADA) criteria [5]. In OGTT, diabetes is diagnosed by blood glucose levels greater than 11 mmol/L, after two hours post ingestion of 75 g of glucose, diluted in water [5, 18, 19]. The OGTT is not always a necessity, except during pregnancy, or for patients who are thought to be diabetic but diabetes unconfirmed using other diagnostic methods [5].

Glycosylated haemoglobin (HbA_{1c}) is a test that is used to measure the level of glucose control[5]. Elevated blood glucose levels bind to the amino-terminal valine of the haemoglobin β-chain, progressively and permanently; hence the measurement of HbA_{1c} assists in determining the level and quality of glucose control over time [5]. Red blood cells have a long half-life120 days [7]; hence the percentage of HbA_{1c} is the measure or index of glucose concentration for the preceding eight weeks, with normal values set to be between 4 to 6% [5, 7]. Values of between 10 to 12% are suggested to reflect poorly controlled glucose levels and treatment goals aim for values of less than 7% HbA_{1c} [19]. The HbA_{1c} test is, however, suggested to have limitations as the observed results can be affected and lowered by diseases such as renal disease and anaemia, or any other disease types that affect the life or longevity of red blood cells [7]; hence often not preferred over blood glucose tests [18]. Other limitations of the HbA_{1c} is that it is not very sensitive and is very expensive [19]; however, it is confirmatory [5, 7, 19].

Urine glucose tests measure the amount of glucose present in the urine, using reagents or dipsticks [5]. These tests are, however, thought to be cumbersome and their results are loosely interpreted as they are prone to many factors [5]. Urine ketone tests use the nitroprusside reaction to test for acetoacetate, however, do not measure β-hydroxybutyrate (which is a major ketone in type 1 diabetic individuals) [5]. Therefore, when ketones are in the form of β-hydroxybutyrate, the urine ketone test may yield negative results in patients with ketosis [5]. These tests may at times be used in combination in order to get accurate results.

1.2.3Type 1 diabetes

Type 1 diabetes is a partially immunologically-mediated disease [4, 20] and is caused by the destruction of islet β-cells in the pancreas [20]. The destruction of the β-cells ultimately results in elevated glucose levels where insulin is required for survival [4], which may lead to

complications such as ketoacidosis, which is associated with low blood pH, profound electrolyte imbalance, resulting from a combination of elevated glucagon levels and insulin deficiency [5]; coma and even death if insulin is not administered [4]. Low or no insulin secretion, is often accompanied by untraceable levels of the C-peptide in the plasma [4].

Insulin signalling causes the translocation of the glucose transporter-4 (GLUT4) to the plasma membrane, allowing for glucose to enter the β-cells [4, 21]. The absence or lack insulin results in the absence of GLUT4 translocation, resulting in reduced or no glucose uptake within the cell. The process of beta cell destruction may at times be detected without any metabolic abnormalities or before the disease is manifested clinically [4, 22]. The presence of insulin, β-cell as well as anti-glutamic acid decarboxylase (anti-GAD) antibodies are usually used to identify the autoimmune processes that lead to the destruction of beta cells and progression of Type 1 diabetes [4]. The rate of beta cell destruction differs among different patients, but has been observed to be faster in children and slower in adults [4, 22, 23].

The slow progression of beta cell destruction, present mainly in adults is known as Latent Autoimmune Diabetes in Adults (LADA). In some patients, particularly children and teenagers, the disease‘s first clinical manifestation is the presence of ketoacidosis. Others may have modest hyperglycemia, which in the presence of other diseases may change rapidly to severe hyperglycemia, and sometimes ketoacidosis [4]. However, some patients, more so adults, may retain some functionality of their beta cells and not develop ketoacidosis for many years [4]. Ultimately all Type 1 diabetes patients become dependent on insulin and are all at a high risk of developing ketoacidosis.

1.2.4 Type 2 Diabetes

Type 2 diabetes, also known as the insulin independent form of diabetes, is adult onset, and individuals suffering from it usually display insulin resistance [4, 18]. The exact and specific causes of Type 2 diabetes are not well defined, but are thought to be a combination of genetic, environmental as well as life style factors[4, 24].Type 2 diabetes commonly remains undiagnosed because the hyperglycaemia remains latent [4], and is often only diagnosed during tests or after the disease has progressed as far as causing polyuria or polydipsia[18, 19]. Type 2 diabetes is diagnosed using the FBG, two hour OGTT, the random blood glucose test as well as the HbA_{1c} test [19], in combination or individually. The disease is characterised by insulin resistance in peripheral tissues, particularly muscle and fat, but also in the liver, leading to increased secretion of insulin and eventually β-cell failure [25]. Insulin resistance often results from obesity and sedentary lifestyle [7, 26]. Both insulin receptor and insulin post-receptor signalling defects have been reported in the progression of insulin resistance, but the primary cause between the two is still unclear [7, 21]. Pancreatic β-cells compensate for insulin resistance for some time, but in some patients the pancreatic cells fail to keep up with the demand for insulin [21]. Although non-diabetic obese individuals are prone to developing insulin resistance and diabetes, some patients who are obese may never develop diabetes, because their β-cells secrete insulin enough to compensate for increased glucose levels [27].In general, Type 2 diabetes patients have circulating insulin levels, but these are often not enough to overcome the insulin resistance in target tissues. This insulin resistance thus result in the loss of β-cells by apoptosis [9, 21]. This then results in defective insulin secretion, particularly in response to post prandial glucose stimuli [9], which leads to imbalances between insulin action and hormones that regulate it [21]. The resulting outcomes of these imbalances are hyperglycaemia and dyslipidaemia, as well as inappropriate mobility of energy in storage in the liver and adipose tissue [28].

Genetic defects can lead to obesity, insulin resistance as well as pancreatic β-cell failure[25-27], and may be monogenic or polygenic [9]. Mature Onset Diabetes in the Young (MODY) results from early β-cell failure, which genetically is caused by an inherited gene mutation in Hepatic nuclear factor-4 [9, 21]. The fact that some diabetic patients do secrete insulin, suggests that Type 2 diabetic individuals can be treated with orally available medications that function to (i) slow down the absorption of sugars in the gastro intestinal tract, thereby reducing blood glucose levels (ii) increase the amount of insulin released by the pancreatic cells and (iii) sensitize or make sensitive target cells to the action of insulin [21]. Due to the aetiology of this disease being multi-factorial, it is most probable that the number of people affected by it will in the near future be reduced, as specific therapies may be discovered [4].

1.3 Aetio-pathogenesis of diabetes mellitus

The core causes of diabetes mellitus are not certainly known, but a number of processes involved in its aetiology are known [3, 4]. Such processes are those involved in the destruction of beta cells of the pancreas, consequently leading to insulin deficiency, as well as other processes that result in the resistance of insulin action [4]. Insulin deficiency or insulin insensitivity leads to carbohydrate, protein and fat catabolism, as a result of the deficient action of insulin on target tissues [4, 5].

Hyperglycaemia, which is the main feature of diabetes, leads to a number of metabolic derailments in diabetic patients [19, 29, 30]. Hyperglycaemia causes alterations in the Na^{+}/Ca^{2+} ion channels resulting in elevated Ca^{2+} intracellular concentrations [29], and also increased production of free radicals and their intermediates, which produce highly glycative, glycoxidative, carbonyl compounds leading to oxidative stress. These play important roles in the pathogenesis of diabetes [31]. These free radicals are formed as a result of Advanced Glycation End-products (AGE) formation, the polyol pathway as well as the activation of protein kinase C and the hexosamine pathway [19], which are the mechanisms by which hyperglycaemia induced damage leads to complications in diabetes [19, 30].

1.3.1 Advanced Glycation End-products (AGE) pathway

Advanced Glycation End-products (AGE‘s) are defined as a varied group of compounds which occur as a result of the non-enzymatic reactions of free amino groups such as lipids, proteins and nucleic acids, with reducing sugars [31, 32]. AGE‘s function by cross linking proteins of the vessel wall, resulting in the thickening and leakage of the vasculature [29]. AGE formation results in plasma proteins binding to AGE receptors on endothelial cells, leading to the formation of Reactive Oxygen Species (ROS), [18, 30, 33], which impair cellular interactions, and also cause damage to vascular function, resulting in endothelial vasomotor dysfunction [29].

1.3.2 Protein Kinase C (PKC) pathway

Protein Kinase C (PKC) is a cytoplasmic phospholipid–dependent kinase, which is activated by calcium [34], belonging to the serine/threonine kinase family [34, 35]. Elevated glucose levels increase the amounts of 1, 2~diacylglycerol (DAG), which is a lipid messenger, leading to the activation of PKC isoforms, causing blood flow alteration in the glomeruli of kidneys and eye retina [30, 34]. The effects of PKC activation in the progression of diabetic vascular complications include blood flow alterations, the thickening of the capillary basement membranes, as well as the expansion of the cellular matrix [30, 35].

1.3.3The Polyol Pathway

The polyol pathway occurs in two metabolic steps where glucose is converted to sorbitol by aldose reductase; and sorbitol to fructose by sorbitol dehydrogenase [30, 36]. Aldose reductase, catalyses the reduction of carbonyl compounds such as glucose, using up NADPH [36] resulting in the induction or exacerbation of oxidative stress, thus increasing hyperglycaemic damage [30, 36]. Sorbitol is oxidised to fructose by sorbitol dehydrogenase, using up NAD [30, 36]. Fructose is phosphorylated to fructose-3-phosphate, which is further broken down to 3-deoxyglucosone [30], which are important glycosylating agents that contribute to the formation of AGEs [36]. Polyol pathway activation alters cellular activity exposes cells to oxidative stress; and contributes to diabetic retinopathy [36].

1.3.4 The Hexosamine Biosynthesis Pathway

The Hexosamine Biosynthesis Pathway (HBP) is the smaller branch of glycolysis [37], during which fructose-6-phosphate is converted to glucosamine-6-phosphate, by the enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) [37, 38]. Glucosamine-6-phosphate is then converted to UDP (uridinediphosphate) *N*-acetyl glucosamine (UDP-GlcNAc) [39, 40], which is used in lipid and protein glycosylation [40]. UDP-GlcNAc attaches to serine and threonine residues of transcription factors, modifying the transcription

factors leading to ―pathologic gene expression changes" [39]. Activation of the HBP results in gene expression modification. This leads to of diabetic complications [30, 39, 40].

Although hyperglycaemia is said to be the earliest and first detectable sign of Diabetes Mellitus (DM), some metabolic errors of diabetes such as blood vessel dysfunction and defects in lipid metabolism are thought to cause alterations in the utilization of substrates, long before elevated sugar levels are detected. Furthermore, when there is a lack of insulin in diabetes mellitus, fatty acids stored in adipose tissue are released, resulting in free fatty acid (FFA) levels being increased. These FFA's then compete with glucose for storage in muscle, as well as for oxidative disposal. This competition further exacerbates hyperglycaemia.

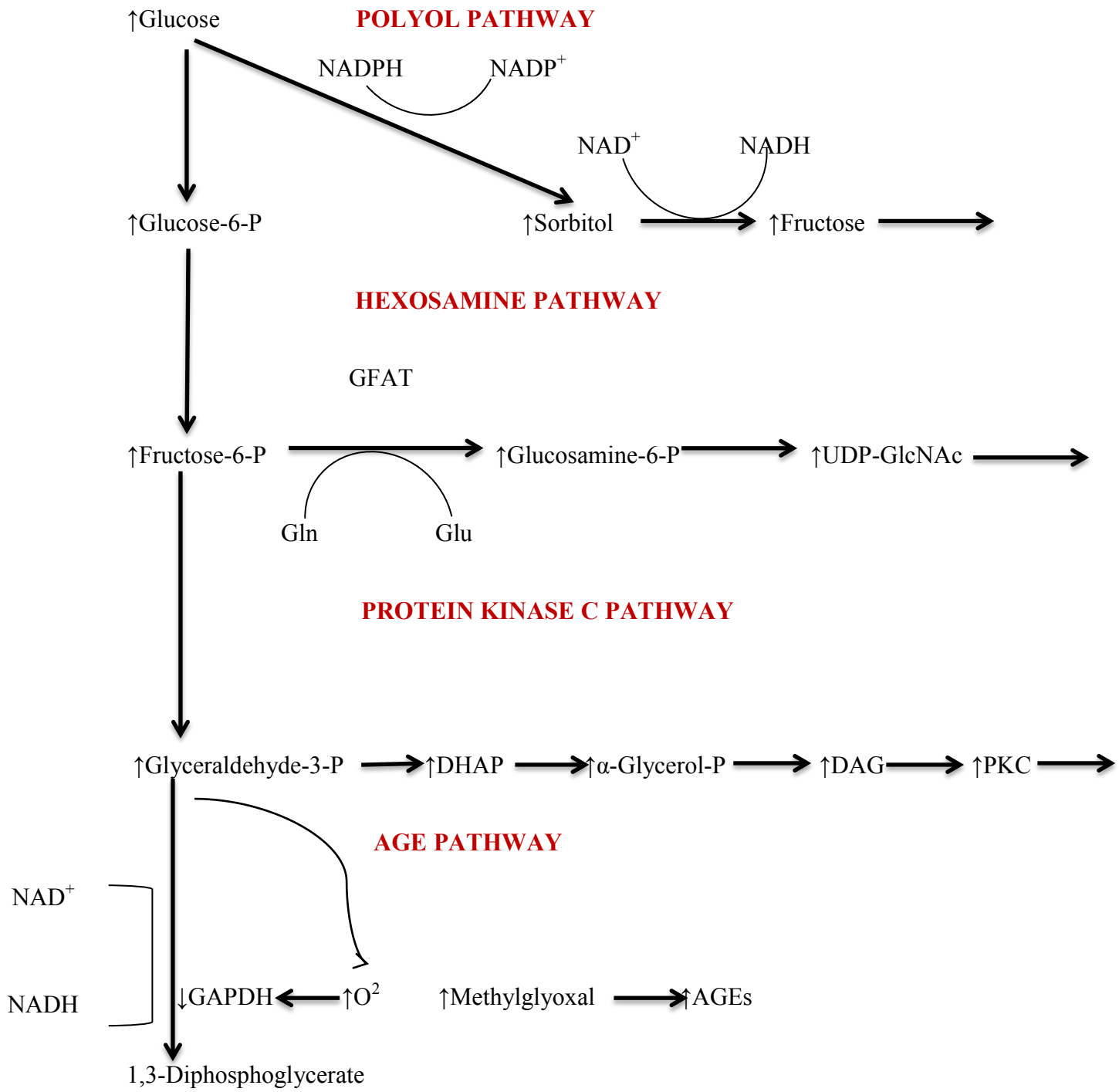


Figure 1: Pathways that lead to diabetic complications include the polyol pathway, the hexasomine pathway, the protein kinase C pathway and the AGE pathway. Adapted from Brownlee, M., 2001 [30].

1.4 Complications of diabetes mellitus

1.4.1 Acute complications of diabetes – Diabetic Ketoacidosis (DKA)

The persistence of hyperglycaemia leads to both acute and chronic complications. One of the major acute complications of diabetes is diabetic ketoacidosis [21]. Diabetic ketoacidosis (DKA) is defined as a complication that results in ketoacidosis and electrolyte imbalances [5], caused by a relative or absolute lack of insulin. This lack of insulin causes hyperglycaemia leading to dehydration and lipolysis [5, 41]. In diabetic ketoacidosis, deficiency in insulin, results in increases in catabolic hormones, that causes the over production of glucose and ketone bodies [8]. In renal tubes, excess glucose draws water, sodium, potassium, magnesium, calcium and other ions into the urine, from the circulation [5]. This osmotic diuresis is what results in dehydration and other electrolyte imbalances associated with diabetic ketoacidosis [5].

DKA occurs more commonly in Type 1diabetic patients, and is said to be a major medical emergency, and still remains the number one cause of mortality in people with Type 1 diabetes [8]. In patients unaware that they are diabetic, DKA occurs over several days, and as dehydration becomes more severe, the brain begins to experience an increase in osmolality [5, 7]. Mental disturbances then follow, beginning with weariness arising from metabolic acidosis [7]. Without treatment or if no care is provided, weariness becomes more severe and may eventually lead to coma [7]. While dehydration is usually the main presenting system of DKA, in some patients, it is acidosis rather than dehydration that is the main presenting system [7, 42, 43]. This often occurs in diabetic patients who are alert, however, with dyspnoea stimulated by acidosis; or in patients who consume large amounts of alcohol or fail to take their insulin dosage [42].

The complications of diabetic ketoacidosis include cerebral oedema, acute respiratory distress syndrome and acute circulatory failure [8], and may lead to death if not treated. Clinically, 95% of patients with DKA have normal total sodium levels and potassium, magnesium and phosphorous deficits are usually masked [5]. In DKA patients, the fasting serum glucose levels are usually greater than 25 mmol/L; occurring as a function of dehydration, which in itself may further elevate fasting serum glucose [7]. The management of diabetic ketoacidosis is multi-faceted, with patient education being very important. Treatments include fluid replacement therapy, and the administration of insulin [42, 43].

1.4.2 Chronic complications of diabetes mellitus

Chronic complications of diabetes are primarily vascular, made up of both microvascular and macrovascular complications [8]. These complications usually lead to morbidity and mortality, and occur late in diabetes (15-20 years) after diagnosis [5]. Microvascular complications of diabetes include retinopathy, nephropathy, peripheral and autonomic neuropathy as well as foot disease [8]. Macrovascular complications include coronary circulation, cerebral circulation, peripheral circulation [8]. Discussed in the following sections are the complications of diabetes mellitus.

1.4.2.1 Diabetic Retinopathy

Diabetic retinopathy is one of the leading causes of blindness, in adults between the ages of 30 and 65, in developed countries [8, 41, 44], and occurs in all diabetes types [45]. This visual loss is associated with increased morbidity, which is often attributed to cardiovascular disease [44]. The severity of diabetic retinopathy ranges from background retinopathy (without maculopathy), which is mild to moderate non-proliferative [7, 8, 44].Background retinopathy has no immediate threat on vision [5, 8]. The next stage of diabetic retinopathy is maculopathy, an illness of the macula, an area vital for vision in the retina [5, 8], followed by

pre-proliferative retinopathy, which is severe or non-severe non-proliferative; proliferative and finally advanced retinopathy [7, 44], which are all sight threatening [5, 8].

In diabetic states, hyperglycaemia causes increased blood flow, directly affecting retinal endothelial cells and pericytes (cells that wrap around the endothelial cells of capillaries and venules throughout the body) [8]. The loss of retinal endothelial cells and death of pericytes results in changes in the integrity of the blood vessels of the retina, as well as impaired vascular auto regulation, such as altered blood retinal barrier [8, 41]. Uncontrolled retinal blood flow leads to endothelial cell proliferation, altered vascular permeability and an increased production of vasoactive substances; resulting in closure of capillaries [8, 41]. This then leads to retinal hypoxia (chronic), and the production of vascular endothelial growth factor (VEGF) is stimulated, which in turn stimulates endothelial cell growth [8]. Endothelial cell growth causes new vessel formation, as well as well increased vascular permeability, which leads to exudative damage [8, 41]. VEGF accomplishes this by acting via protein kinase C [8, 41].

Clinically, the onset or background of diabetic retinopathy is characterised by features such as microaneurysms, haemorrhages, exudates, cotton wool spots, intra microvascular abnormalities, venous changes, as well as neovascularisation and ischemia [46]. Microaneurysms, haemorrhages and hard exudates are said to be the background of retinopathy, as their occurrence is very common in diabetic retinopathy [45].

After diagnosis of diabetes, if good glycaemic control is maintained, diabetic retinopathy can be prevented [41], and the severity of the disease can be related to the quality of glycaemic control [5]. Hyperglycaemia is known to promote hyper-perfusion of the eyes; therefore a fast reduction of blood glucose may initiate deterioration of retinopathy, causing relative ischemia [8, 41]; hence, glycaemic control should be gradual. Regular screening for diabetic

retinopathy is also a good preventive measure for all diabetes patients, but more so for those at risk of developing diabetic retinopathy [5, 7, 8]. Other measures to prevent diabetic retinopathy include maintenance of glycaemic control, however, avoiding stress caused by hypoglycaemic reactions, and the control of blood pressure [7].

Both proliferative and non-proliferative retinopathies are treatable with retinal photocoagulation (condensation of protein material by the use of intense beam of light, in a controlled manner [5, 8]. Photocoagulation assists in the destruction of retinal ischaemia and reducing the production of growth factors such as VEGF [41]. Photocoagulation is also used as a seal for leaking microaneurysms, assists in the reduction of macular oedema and to obliterate new vessels on the retinal surface [8]. Tight diabetic and optimal blood pressure (BP) controls are known to reduce diabetic retinopathy and are among key treatments [7]. Furthermore, it is recommended that both Type 1 and Type 2 diabetics be examined for retinopathy every year [7, 41].

1.4.2.2 Diabetic Nephropathy

One of the leading causes of morbidity and death, and is a leading cause of end-of stage renal failure/disease (ESRF or ESRD) in developed countries[44]. Diabetic nephropathy occurs together with other microvascular as well as macrovascular complications of diabetes, and requires early intervention [8]. The progress and development of diabetic nephropathy is thought to be very complex because of the number of different cell populations found in the kidney and also because the kidney plays many different pathophysiological roles [41]. High glucose concentrations are the initiating factor, inducing several cellular effects, involving a diverse types of cells, such as endothelial cells, smooth muscle cells, masangial cells, podocytes (glomerular epithelial cells) and inflammatory cells [41]. Diabetic nephropathy risk factors include but are not limited to poor glycaemic control, hypertension [41, 44], as well as the duration of diabetes and family history of diabetic nephropathy [8, 44].

The factors that interplay in the pathogenesis of diabetic nephropathy include metabolic changes, hemodynamic changes [41, 44], as well as genetic and environmental factors [44]. Metabolically, serum AGE levels that occur as result of hyperglycaemia have been found in nephropathic kidneys [44]. Hemodynamic changes occur early in diabetic nephropathy and are often characterised by glomerular hyper-filtration [41, 44].

One of the strongest determinants of glomerular hemodynamic changes and pressure is renal Angiotensin II (ANG II) [47]. ANG II is the principal factor in the renin-angiotensin system (RAS), which is the "co-ordinated cascade of proteins and peptide hormones" [48]. In diabetic nephropathy, there is an increased production of ANG II within the kidney; in spite of the suppression of general RAS [48].This increased production of ANG II contributes to the exacerbation of diabetic nephropathy through a number of hemodynamic, tubular and growth promoting actions [48]. Clinically, diabetic nephropathy starts with the thickening of the glomerular basement membrane, and the accumulation of matrix material in the masangium [8]. This is followed by nodular deposits, and hardening of the glomerulus of the kidney (glomerulosclerosis) [8]. Eventually the glomeruli are lost and the function of the kidney declines.

Upon symptoms and/or diagnosis of diabetic nephropathy, strategies to improve glycaemic control, reduce blood pressure and provide Angiotensin Converting Enzyme Inhibitor (ACE-I), should be put in place to arrest progression [8]. ACE inhibitors appear to be more beneficial in controlling diabetic nephropathy than BP [8]. Angiotensin II receptor antagonists have been shown to yield the same results, but with additional risk of potassium increase in diabetic cardiomyopathy [8]. Although it comes with its own challenges, renal transplantation is beneficial more at early stages rather than in patients with ESRF [8].

1.4.2.3 Diabetic Neuropathy

Diabetic neuropathy is described as a disease that affects both the somatic and autonomic parts of the peripheral nervous system, however, there is evidence that the upper central nervous system and spinal cord are also affected in the long run [41]. Diabetic neuropathy occurs secondarily to metabolic disturbances and its prevalence is directly proportional to the extent of diabetes and glycaemic control [8, 49], although an early onset severity has been described [45]. There are two classes of diabetic neuropathies, namely diabetic polyneuropathy, where diabetes causes diffuse damage of the peripheral nervous system; as well mononeuropathy, where diabetes causes focal damage [49]. Symmetrical sensory polyneuropathy is the most common form of diabetic neuropathy [45], and its other forms include cranial, peripheral motor neuropathy and autonomic neuropathy [49]. Focal or mononeuropathies include Carpal Tunnel Syndrome (CTS) and Ulnar Neuropathy at the Elbow(UNE) [49]. Both polyneuropathy and mononeropathy play significant roles in sensory and motor deficits and are both associated with disability in patients [49]. Autonomic neuropathy which is not necessarily associated with peripheral somatic neuropathy [8], affects gastric and intestine the movement and activity, erectile function, function of the bladder, vascular tone and cardiac function [45].

Diabetic neuropathy tends to occur early in diabetes and is often without symptoms in the majority of patients, but can cause major disability problems in others [50]. Pathologically, diabetic neuropathy manifests in the degradation of axons of myelinated and unmyelinated fibres, the thickening of Schwann cell basal lamina, demyelination that is segmented and patchy, as well as the thickening of the basal membrane and microthrombi in intraneural capillaries [8]. Endothelial enlargement with reduced oxygen and tension and hypoxia are also observed characteristics in the progression of diabetic neuropathy [41].

Medications designed to inhibit the renin-angiotensin system as well as α1-antagonists are useful in improving nerve conduction velocities, resulting in increased neural blood flow [41]. The pathophysiology and therapy of diabetic neuropathy is not fully understood [49].

1.4.2.4 Macrovascular complications

Diabetes increases the risk of cardiovascular events and deaths in affected individuals [45]. One of the main differentiating characteristics of cardiovascular events in diabetics as opposed to non-diabetics, is the higher frequency of coronary artery disease [51], due to elevated hyperglycaemia. Coronary artery disease tends to occur at younger ages in diabetic individuals, especially in the presence of renal diseases [45]. More than 50% of all diabetics die from cardiovascular disease related diseases [52]. While the exact mechanisms that lead to cardiovascular disease are not fully understood, they involve the formation of atherosclerotic plaques or atherosclerosis, which leads to death in both type 1 and type 2 diabetes [52, 53].

A third of all diabetics develop aggressive complications, and more than 70% of all diabetics die of atherosclerotic-related diseases [52, 53]. Atherosclerosis is an inflammatory disease, characterised by the thickening of artery walls due to fatty material or lipid accumulation [54-56]; and is related to and often leads to cardiovascular disease [54, 55]. This accumulation of lipids in the arteries affects the supply of the blood to the heart, brain as well as the lower parts of the body [30]; hence diabetic patients are at increased risks of strokes, heart attacks and lower limb amputations [30, 54, 55]. Endothelial dysfunction is hypothesised to be the first step in the progression of atherosclerosis arising from hyperglycaemia, increased and oxidised low density lipoprotein (LDL) levels, genetic factors, and free radicals [56].

Atherosclerosis is not a distinct feature of diabetes mellitus, but is worsened by hyperglycaemia [45]. Insulin resistance and hyperglycaemia reduce the production of nitric oxide (NO) [30, 57], an important vasodilator of the endothelium [57]; and increase the proliferation of the vascular smooth muscle cells [30]. Vasodilation of the endothelium occurs when there is cholinergic input into the endothelial cells, activating NO synthase (NOS) resulting in the increased production of NO from its precursors [57]. In a disordered or

dysfunctional epithelium, acetylcholine acts on themuscurinic receptors on the smooth muscles, causing vasoconstriction [57].

Responses that change the normal course of endothelial function are triggered, with endothelial permeability and adhesiveness, with respect to leucocytes and platelets, being increased [56]. A host of inflammatory responses are triggered, further thickening the artery wall. Macrophages and T-lymphocytes which are monocyte derived mediate these inflammatory responses [56]. Eventually the artery cannot compensate the dilation, allowing the lesion to intrude into the lumen and blood flow is altered [56, 57].

Another common cause of injury or dysfunction of the endothelium and underlying smooth muscle is Low Density Lipoprotein (LDL) and lipoprotein modifications [28, 56]. When LDL‘s are trapped in the artery, they are engulfed by macrophages as oxidation progresses. This leads to the formation of cholesterol esters and lipid peroxides within the artery, resulting in the formation of foam cells. The vicious cycle of inflammation, lipoprotein modification and further inflammation, leading to the intrusion of the artery into the lumen and obstructing blood flow, is maintained by the presence of these lipids in the artery [56]. All these result in the thickening of the endothelial basement membrane.

The treatment of atherosclerotic events such as hypertension and hyperlipidaemia tends to have greater macrovascular impact in diabetic individuals, than glucose control [58]. This couldimply that the atherogenic effects observed in diabetic patients could be because of the macrovascular complications such as hypertension and dyslipidaemia, and not the impaired glucose function [56].

1.5 Diabetic dyslipidaemia

While hyperglycaemia is thought to be the most significant feature in the progression of microvascular complications in diabetic patients, dyslipidaemia is an equally significant feature in the progression of macrovascular complications in diabetic patients [28]. Atherogenicdyslipidaemiais a common feature in diabetics, especially type 2 diabetics, and is defined by three main features; elevated Low Density Lipoprotein (LDL) cholesterol, decrease High Density Lipoprotein (HDL) cholesterol, as well as increased Very Low Density Lipoprotein(VLDL)-triglycerides in the blood, leading to cardiovascular disease [3, 11, 28, 59, 60]. Predisposing features to atherogenicdyslipidaemia include obesity, the metabolic syndrome, insulin resistanceas well as alterations of insulin promoted fatty acids uptake in the skeletal muscle, and genetic factors [3, 52, 53, 59, 60].

The exact pathogenesis of diabetic dyslipidaemia is not known [61], but factors that are thought to be the probable causes include the effects of insulin on liver apoprotein production, regulation of Lipoprotein Lipase (LpL), the actions of insulin on adipose and muscle tissues; as well as the Cholesterol Ester Transfer Protein (CETP) actions [58]. Furthermore, these features are promoted by insulin resistance [28].

In type 2 diabetes, insulin resistance is associated with elevated levels of insulin in serum, as well as in the decrease in the number of β-cells. This results in an altered regulation of lipoproteins in circulation, as well as altered glucose levels [28]. Fat cells that are insulin resistant release free fatty acids (FFA), leading to an increase in FFA‘s that enter the liver, promoting the production of triglycerides (TG) [61]. This inability of insulin to suppress the production of VLDL-triglycerides in the liver of patients with type 2 diabetes (as a result of insulin resistance), results in the increase of plasma TG levels [28, 61] and stimulates the secretion of alipoprotein-B (AlipoB) [61]. Furthermore, free fatty acids circulating in the

blood stream increase, leading to an even greater degradation of stored triglycerides in adipose cells [59]. Increased levels of VLDL-triglycerides results in the reduction of HDL-C, as the triglycerides are transferred to HDL upon collision of these particles [28]. This reduction of HDL-C levels is followed by reduced anti-oxidant and anti-atherogenic activitiesof HDL [28, 61]. Furthermore, increased VLDL-triglycerides result in increased small dense LDL-C particles concentrations [28, 61], through the CETP action [58, 61]. When the LDL-C particles become smaller and denser, they become more susceptible to oxidation and are more readily invade the walls of arteries, contributing to atherosclerosis [28].

Acyl-CoA: Cholesterol Acyltransferase (ACAT) and 3-hydroxy-3-methylglutaryl coenzyme (HMG-CoA) reductase are key enzymes in the regulation and synthesis of plasma cholesterol, respectively [62, 63]. ACAT is located in the rough endoplasmic reticulum of cells and occurs in two isoforms, ACAT-1 (expressed in most tissues) and ACAT-2 (expressed mainly in the intestine and liver) [64]. ACAT is involved cholesterol esterification, cholesterol absorption as well as the accumulation of cholesterol in the arterial walls [65]. This accumulation is one of the main metabolic changes that occur in an atherosclerotic lesion [66] and the elevation of intestinal ACAT activity contributes to dyslipidaemia induced by diabetes [67]. HMG-CoA reductase is an endoplasmic reticulum-bound, rate limiting enzyme in the biosynthesis of cholesterol [64, 68]. HMG-CoA regulates cholesterol metabolism as well as plasma cholesterol concentrations [68].

1.5.1 Treatment of diabetic dyslipidaemia

Treating diabetic dyslipidaemia is likely to reduce cardiovascular events in diabetic individuals by up to 50% [58], and the goal of therapy is the reduction of LDL-C levels, increasing of HDL and reduction of triglycerides [28]. The American Diabetes Association (ADA) recommends LDL cholesterol levels lower than 100 mg/dL (2.60 mmol/L), HDL

cholesterol levels more than 45 mg/dL (1.15 mmol/L) as well triglycerides levels of no more than 200 mg/dL (2.3 mmol/L) [58]. This was modified in 2004 to target cholesterol levels of HDL > 40 mg/dL (1.0 mmol/L) in men and HDL 50 mg/dL (1.3 mmol/L) in women [69]; as well triglycerides levels < 150 mg/dL (1.7 mmol/L). Although LDL levels do not form part of the aberrations that lead to diabetic dyslipidaemia[11], LDL lowering recommendations are made on the observation that adult diabetics without any macrovascular disease, usually have the same propensity of developing cardiovascular disease as non-diabetic patients who have had cardiac event/s [58].

Attaining desired plasma lipoprotein and lipid levels often requires pharmacotherapy combined with lifestyle modifications (diet and exercise) [28, 61]. Dyslipidaemia is currently treated with lipid lowering agents, such as statins, niacin, ezetimibe, fibrates, nicotinic acid, bile acid sequestrants and omega-3 fatty acid [28, 61], in addition to good glycaemic control [28]. ACAT and HMG-CoA reductase inhibitors also play vital roles in lowering cholesterol levels as well as being anti-atherosclerotic agents [3, 63]. The common HMG-CoA reductase inhibitors include statins such as simvastatin [3].

Table 1 A list of diabetic dyslipidaemia treatments, with their efficacy, adverse events and specific agents. Reproduced and adapted from Mooradian, AD [61].

Drug	Efficacy	Adverse Effect	Specific Agents
Statins	LDL↓18-55% HDL↑5-15% TG↓7-30%	Hepatoxicity, myopathy, ↑creatine kinase levels	Rosuvastatin; fluvastatin; simvastatin; pravastatin; atorvastatin; lovastatin, extended release lovastatin
Ezetimibile	LDL↓15-20% HDL↑1% TG↓8%	No major adverse effects	Ezetimibile
Ezetimibile + Simvastatin	LDL↓30-60% HDL↑9% TG↓20%	Hepatoxicity, myopathy, ↑ creatine kinase levels	10/10 mg to 10/80mg orally once daily
Nicotinic Acid	LDL↓5-25% HDL↑15-35% (ApoA-l↑) TG↓20-50% Small dense LDL↓	Hot flashes, hyperglycaemia, hyperuricemia, hepatoxicity	Nicotinic acid; extended release nicotinic acid;sustained release nicotinic acid
Nicotinic Acid + Statin	LDL↓30-42% HDL↑20-30% TG↓32-44%	Hot flashes, hyperglycaemia, hyperuricemia, hepatoxicity ; myopathy, ↑ serum creatine kinase levels	Lovastatin and nicotinic acid; extended releasenicotinic acid and simvastatin
Fibrates	LDL↓5-20% HDL↑10-15% TG↓20-50% Small dense LDL↓	Dyspepsia, gallstones, hepatoxicity, myopathy	Fenofibrate, micronized; fenofibrate; bezafibrate;gemfibrozil; modified-release bezafibrate
Bile acids sequestrants	LDL↓10-20% HDL↓1-2% TG possible↑	Gastrointestinal distress,constipation	Colestyramine; colestipol hydrochloride;colesevelam hydrochloride.
Omega-3 fatty acid	LDL↑5-10% HDL↑1-3% TG↓25-30%	Fishy aftertaste, gastrointestinal disturbances	Omega-3-acid ethyl esters; over the counter fish oil; dietary supplements containing omega-3 fatty acids; EPA and DHA

Abbreviations: ↑-increase; ↓-decrease; EPA – eicosapentaeonic acid; DHA-decosahexaenoic acid

1.5.2 Lipid lowering drugs

Statins

Statins are recommended as first-line pharmacological treatment for diabetic dyslipidaemia [61]. They have been shown to be effective in lowering LDL-C levels by 18-55%, elevating HDL-C by 5-15%, and reducing TG levels by 7-30% [28, 61]. Their use is limited by side effects such as myopathy, gastrointestinal upsets, dose-dependent hepatoxicity and myotoxicity [28].

Other lipid lowering Drugs

Other lipid lowering drugs used in the treatment of diabetic dyslipidaemia include niacin, ezetimibe, fibrates, nicotinic acid, bile acid sequestrants and omega-3 fatty acid [61].The targeted goal of increasing HDL cholesterol levels to more than 40-50 mg/dL in diabetic patients, is more challenging than that of reducing LDL levels [58]; more so in patients with diabetic dyslipidaemia who sometimes report with HDL cholesterol levels of < 35 mg/Dl [58]. Niacin has been shown to be predominantly effective in raising HDL-C levels, with the ability of increasing HDL levels by up to 25%, although higher doses of niacin may worsen hyperglycaemia [28].

Ezetimibe, is a selective _cholesterol absorption inhibitor' which has been shown to be highly effective in reducing LDL-C levels, especially in patients who cannot tolerate statins [28]. Fibrates have been shown to be effective in the lowering of TG and non HDL-C levels, while elevating HDL-C levels [28, 58]. The efficacy, adverse effects and specific agents and their doses of the above mentioned drugs is detailed in Table 1.

1.5.3 Hypoglycaemic agents

Some anti-diabetic agents are claimed to be having lipid lowering effects, more especially TG levels [28]. Metformin has been suggested to be an effective LDL-C, total cholesterol (TC) and TG lowering and an effective HDL-C elevating agent [28]. Pioglitazone, also an anti-diabetic agent, has been shown to be effective in reducing TG levels and increase HDL-C levels [28]. While some of these agents may be effective as monotherapy, combination therapy may yield better outcomes [28].

1.5.4 Combination therapy: lipid lowering and glucose lowering drugs

An example of a highly effective combination therapy of lipid lowering and glucose lowering agents is that of drugs that control bile acids, which may have effect in the metabolism of both cholesterol and glucose levels [28]. Colesevelam hydrochloride, which is a bile-acid sequestrant, has a 10-20% LDL-C lowering effects when used alone [61], and 48% when used in combination with statins, especially in patients with mild-moderate hypercholesterolemia [28]. The use of fibrates together with statins though is known to cause rhabdomyolysisand caution should always be taken when these are used [58]. These are, however, rarely used.

For effective therapeutic outcome, treatments for dyslipidaemia should parallel good glycaemic control. Figure 2 highlights some of the end-points associated with diabetes mellitus.

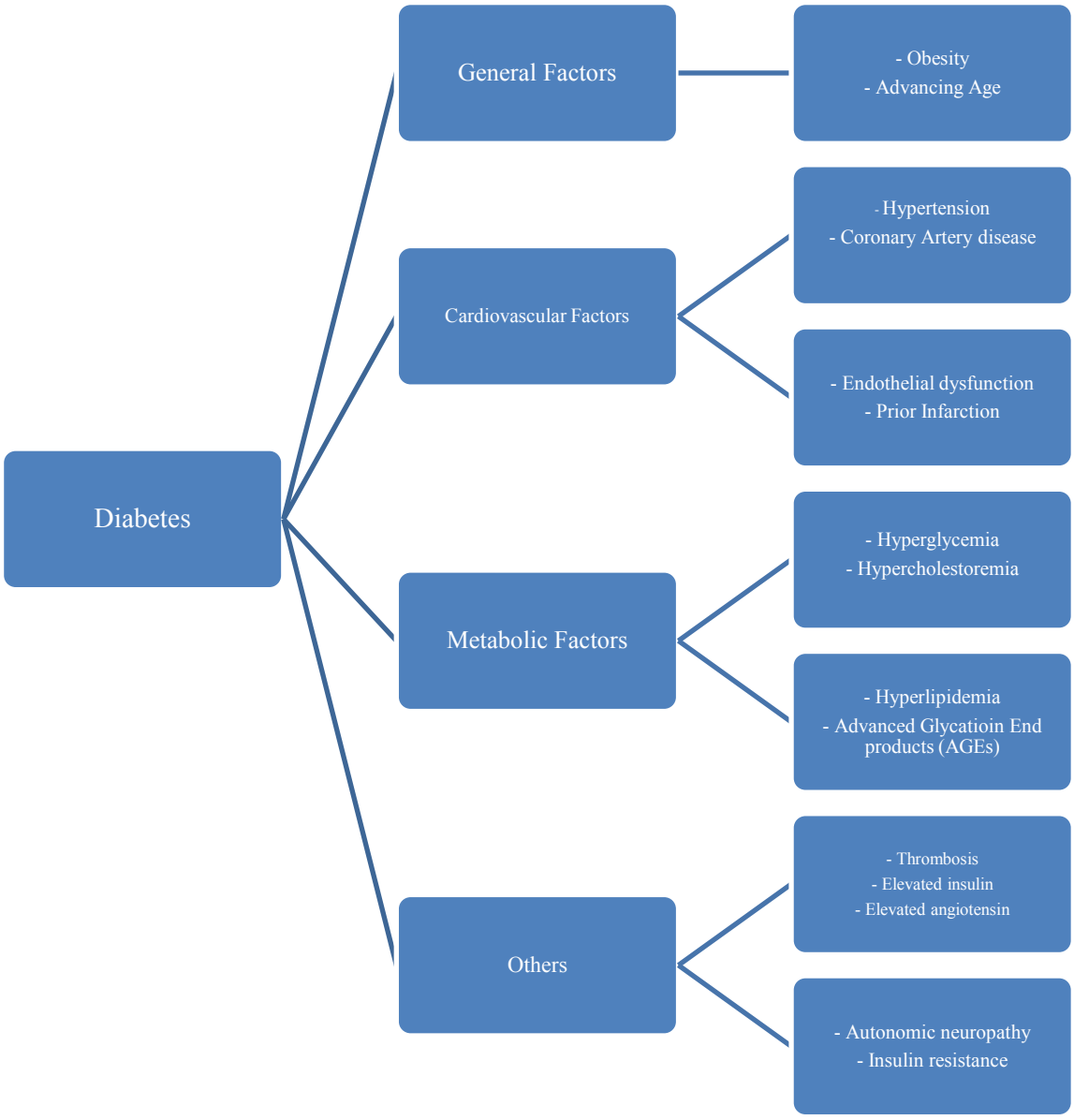


Figure 2: End-points associated with Diabetes Mellitus. Adapted from Wang *et al*., [70]

1.6 Treatment/s or management of diabetes mellitus

Pharmacotherapy

Type 1 diabetes is pharmacologically treated by the administration of sufficient amounts of exogenous insulin [24].If appropriate treatment is applied, it not only achieves euglycaemia but may also result in the desired effects of insulin, such as the reversal of the breakdown of amino acidsand lipolysis in skeletal muscle and ketogenesis in the liver [21].

The treatment of Type 2 diabetes is multi-faceted, since that its aetiology is also multi factorial [4]. Priority in type 2 diabetes treatment is lifestyle modifications. Obese patients should strive to reduce body weight, as well as exercise in order to increase insulin sensitivity [21, 24]. Pharmacological treatments for type 2 diabetes consist mainly of orally available treatments such as α-glucosidase inhibitors, sulfonylureas and meglitinides; as well as thiazolidinediones and biguanides [71, 72]. Metformin is the first-line treatment , but sometimes a combination of these drugs may be used for Type 2 diabetes patients [4]; however, some patients eventually require the administration of exogenous insulin [71].

1.6.1 Insulin therapy

Type 1 and type 2 may need the exogenous insulin for treatment to reduce increased glucose levels [73]. Insulin promotes the uptake of glucose, amino acids and fatty acids. It also increases glycogen synthesis and storage in the liver from glucose [17]. Insulin therapy is initiated in patients who have severe hyperglycaemia, but glycaemic control could not be reached with two or more other treatments [73]. The different types of insulin include rapid-acting (begins to work 15 minutes after injection), regular/short-acting (reaches blood stream 30 minutes after injection), intermediate-acting (gets to work 2 hours after injection), and long-acting (which reaches blood the blood stream after several hours and lowers glucose evenly over 24 hours)insulins [73, 74]. The use of insulin in the treatment of diabetes mellitus

is associated with fewer cases of hypoglycaemia and weight gain, compared to other treatments [75].

1.6.2Intestinal glucose absorption inhibitors: α-glucosidase inhibitors

Alpha glucosidase inhibitors, are also known as starch blockers, in relation to their ability of binding more tightly to α-glucosidase enzymes, in comparison to dietary carbohydrates[24]. Glucosidases function by cleaving to complex carbohydrates resulting in the release of glucose. α-Glucosidase inhibitors therefore increase time for the hydrolysis of carbohydrates by inhibiting these glucosidases [24, 72].α-Glucosidase inhibitors therefore reduce postprandialpeak of blood glucose, and are therefore very effective when taken with meals [21, 76]. They are, therefore, useful as treatment for postprandial hyperglycaemia as well as newly diagnosed patients with mild hyperglycaemia [24, 76]. The common side effects of α-Glucosidase inhibitors include abdominal discomfort, bloating, flatulence and diarrhoea, which all result from gas released by bacteria that metabolise undigested carbohydrates that reach the large intestine [72, 76]. These side effects do, however, moderately become less severe and subside with continued treatment, but contraindicate these drugs in patients with inflammatory bowel disease bowel disease [21, 24].

1.6.3 Insulin secretagogues

The two most commonly used insulin secretagogues are sulfonylureas and meglitinides [21, 24]. These drugs both stimulate and enhance the release of insulin from pancreatic β-cells, which results in increased circulating plasma insulin [24, 71]. Sulfonylureas function by inhibiting the K^+/ATP channels of the β-cell [72], at the SUR 1 sub unit [21]. This results from the increased glucose metabolism causing β-cell accumulation of intracellular ATP, depolarization of the membrane, influx of Ca^{2+}, as well as the fusion of the plasma membrane with vesicles containing insulinwhich gets secreted [72]. Sulfonylureas are generally safe, orally available drugs that are metabolised in the liver [24]. One of their adverse reactions is

that they may cause hypoglycaemia and weight gain, hence are undesirable in obese patients [21, 72].

Meglitinides are very similar to sulfonylureas in their absorption and metabolism, as well as their side effects [21]. Furthermore, meglitinides stimulates the release of insulin by binding the SUR 1 subunit, inhibiting the β-cell K^+/ATP channel; in a similar fashion as sulfonylureas [21, 72]. Although they yield the same results, these drugs bind to different parts of the SUR 1 molecule [72].

1.6.4 Insulin sensitizers

Insulin sensitizers increase the sensitivity of target tissues to insulin. The two most commonly used insulin sensitizers are thiazolidinediones and biguanides [71].

Thiazolidinediones (TZD)

Peroxisome proliferator-activated receptor (PPAR)-γ agonists [thiazolidinedione (TZDs)] expand subcutaneous adipose tissue and secondarily reduce intra-myocellular lipids (IMCLs) and intra-hepatocellular lipids and insulin resistance [71, 72, 77]. These drugs do not affect the secretion of insulin (unlike insulin secretagogues), but rather enhance insulin effects on target tissues [21]. The nuclear hormone receptor, PPAR-γ is expressed in adipose tissues and is involved primarily in the differentiation of adipocytes [21, 77]. It has been shown that patients that have an over expression of PPAR-γ accumulate high levels of adipocyte markers when treated with TZDs. TZDs not only improve insulin sensitivity in adipose tissue, but also in the liver. The improvement of insulin sensitivity in the liver by TZDs is said to be a mystery considering that PPAR-γ is expressed in very minute levels in liver tissues. TZDs improve insulin sensitivity in adipose tissue, by causing changes in adipocyte gene expression, indicated by PPAR-γ, leading to changes in fat metabolism; and subsequently improving insulin sensitivity [21, 77]. TZDs/PPAR-γ mediated changes not only improve

Type 2 diabetes, but are also beneficial in improving conditions that are associated with insulin resistance and hyperinsulinemia, such as polycystic ovarian syndrome [21, 72, 77]. Side effects of TZDs include water retention, which may lead to oedema as well as cardiovascular risks [21].

Biguanides

Biguanides (metformin) function by increasing insulin sensitivity of target tissues, just like TZDs [21, 72, 77] and inhibit the breakdown of fatty acids, and inhibiting hepatic gluconeogenesis, hence promote glycolysis [24]. They achieve this by activating the AMP-dependent protein kinase (AMPK)[21]. Metformin is the first line treatment for type 2 diabetes and it is suggested that metformin prevents AGE formation and is also used in the treatment of Polycystic Ovary Syndrome (PCOS) [78].

The most common side effects of metformin are those of mild gastrointestinal distress, which, however, are minimised by titration of the dosages [21, 72]. The other, more serious effect of metformin is lactic acidosis, which is however, low and predictable [72]. The precautions to be taken with the administration of metformin is that it should not be given to patients with heart failure, hypoxemia, respiratory disease, incidences of ketoacidosis, renal disease, severe infections as well as alcohol abuse [21]. Furthermore, metformin does not affect the secretion of insulin, hence is not associated with hypoglycaemia [72].

Other therapeutic interventions for Type 2 diabetes include incretinmimetics, glucagon-like peptide (GLP)-1 agonists and dipeptidyl peptidase (DPP)-4 inhibitors which increase insulin secretion, and cause weight gain[71].

1.7 Medicinal plants

Bioactive compounds in citrus fruits and juices are thought to have beneficial effects in diabetes mellitus and its related conditions, such as cardiovascular disease and dyslipidaemia.

Citrus fruits are the most consumed fruits worldwide after mangoes, tomatoes and bananas [79]. Citrus fruits are known to contain phytochemicals and phenolics, which are thought to promote good health [79]. These phytochemicals and phenolics include flavonoids, flavones and flavanols, and have been shown to have anti-atherogenic, anti-inflammatory and antioxidant properties [55, 80-83]. The grapefruit is the most common citrus fruit and its consumption thought to induce hypoglycaemic effects, as well as improve lipid profiles in [3, 84, 85].

1.7.1 The grapefruit

C. paradise Macfad., commonly known as the grapefruit, originated in the West Indies, and has been recently introduced to many Pacific islands and other parts of the world [86]. In their study, de Moares*et al.,* [87] reported that the grapefruit got its scientific name, *C. Paradisi,* from Macfadyen in 1830 in reference to a citrus tree known as ‗the forbidden fruit' in Jamaica.The grapefruit is one of the *Citrus* biotypes in which its hybrid origin and subsequent selection for mutants, is well documented [88]. Data from RAPD and SCAR markers indicate that the grapefruit was derived from the backcross between a sweet orange (*C. sinensis*) and pomelo (*C. maxima/ C. grandis*) [87-89].Grapefruit is commercially important as a crop not only because of its juice, but also because it is an important source of oils and pectin [87].

The grapefruit and its juice are thought to play vital roles in the reduction of blood sugar levels in normal individuals, as well as having beneficial effects on other degenerative

diseases [85]. Regular consumption of grapefruit has been shown to be associated with weight loss, particularly in patients with the metabolic syndrome [89].

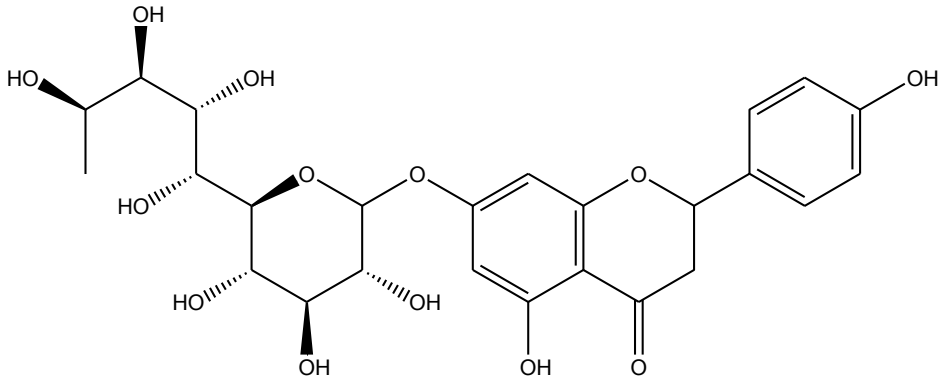
1.7.2 Bioactive constituents of grapefruit

The major grapefruit pharmacologically active compounds include furanococoumarins (bergamottin and 6‘, 7‘ dihydroxybergamottin), as well as the flavonoids hesperidin, naringin and naringinin (Figure 3) [90]. Flavonoids are polyphenols present in abundance in fruits, vegetables and other plant materials [81, 91, 92]. Their primary role in plants is protection against injury, giving plants their colour, as well as function as antioxidants [83, 91].

Upon consumption, naringin is digested and hydrolysed into naringenin, its aglycone, by intestinal bacteria [93, 94]. It is believed that naringenin, rather than naringin, is what bring about the reduction in blood sugar levels [85]. Naringenin is thought to reduce blood sugar levels by suppressing hepatic glucose production [95], as well by increasing muscle cell glucose uptake via AMPK [96].

Other bioactive compounds present in grapefruit include limonoid aglycones, glucosides, ascorbic acid, folic acid, lucaric acid, carotenoids, pectin and potassium [85]. The number of bioactive compounds present in grapefruit and other fruits are have not been fully quantified [85, 90, 96].

a)



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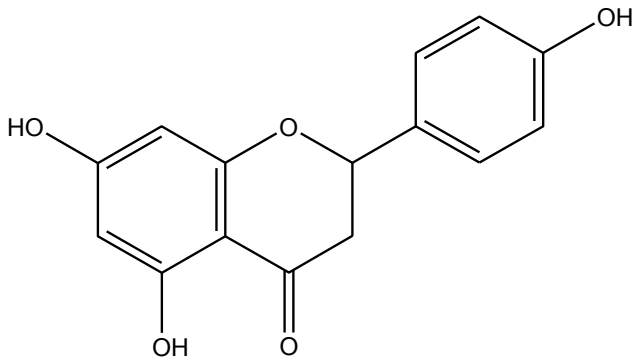

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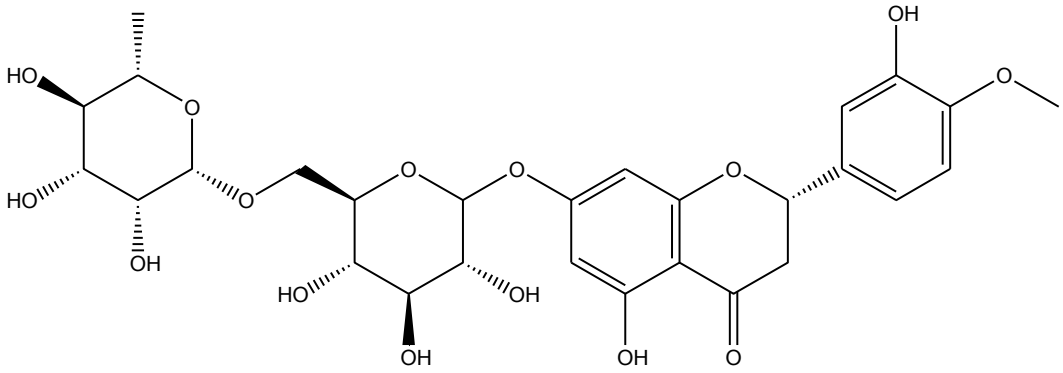


Figure 3: Chemical structures of some of the active polyphenolics present in grapefruit and grapefruit juices. a) Naringin, the most abundant flavoinoid in grapefruit that gives it its bitter taste, b) Naringenin, the naringinaglycone [85], c) Hesperidin.

1.7.3 Grapefruit juice-drug Interactions

Grapefruit juice-drug interactions have been widely reported [88, 94].The inhibition of the intestinal cytochrome P450 3A4 (CYP3A4) enzyme (which metabolises many drugs) is the primary mode by which grapefruit interacts with drugs [90, 97]. Interaction of grapefruit juice with certain drugs causes a significant increase in the plasma concentrations of these therapeutic agents [85, 97]. The inhibition of CYP3A4 is irreversible and the inhibited/inactivated enzyme is destroyed [85, 97]; resulting in high oral availability of drugs that are metabolised by this enzyme [85, 90, 97]. Grapefruit juice interacts only with the intestinal and not hepatic CYP3A4 [97], and therefore only affects orally and not intravenously administered drugs [85, 97]. Grapefruit juice also inhibits other cytochrome P450 enzymes [85], and drug transporters such as P-Glycoprotein (P-gp) and Organic Anion-Transporting Protein (OATP) [85, 94]. Some of the cardiovascular drugs that are also substrates of P-gp are simvastatin, lovastatin, carvedilon and veramapil [85].

Clinical significance of GFJ-drug interactions is not fully understood. Many diabetic patients routinely consume GFJ in anticipation of improved outcome. However, the effects of GFJ consumption on the glycaemic index and lipid profile in a diabetic state have not previously been investigated.

1.8 Hypothesis, aims and objectives

1.8.1 Hypothesis

Grape fruit juice improves glycaemic index and lipid profile in a diabetic state.

1.8.2 Aim

To investigate the anti-diabetic and anti-dyslipidaemic effects of grapefruit juice in induced type-1 diabetes

1.8.3 Objectives

i. To establish a diabetic rat model

ii. To investigate hypoglycaemic effects of grapefruit juice in a diabetic state

iii. To investigate anti-dyslipidaemic effects of grapefruit juice in a diabetic state

iv. To determine the mechanisms by which grapefruit juice exerts hypoglycaemic and anti-dyslipidaemic effects

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

Chemicals and reagents were sourced as indicated below.

Regular insulin, normal saline, portable glucometers and glucose test strips (Bayer AG, Leverkusen, Germany); and were purchased from a local pharmacy. Reagents and chemicals purchased from Sigma-Aldrich were as follows: D-Glucose, streptozotocin, citrate and phosphate buffers, EDTA, anthrone, H_2SO_4, glucose-6-phosphatase, molybdic acid, sodium docecylsulphate (SDS), ascorbic acid, HEPES, KCl, dithiothreitol, $MgCl_2$, inositol diphosphate (IDP), NADH, phosphoenolpyruvate, $Na_2CO_3$and malate dehydrogenase. The primary antibodies, HMG-CoA reductase (C-1) mouse monoclonal antibody (to be diluted 1:1000), insulin receptor substrate 1 rabbit polyclonal antibody (IRS1; to be diluted 1:1000), and ACAT-2 (N-15) goat polyclonal antibody (to be diluted 1:1000) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The secondary antibodies Horse Radish-conjugate goat anti-mouse and horseradish peroxidase-conjugate mouse anti goat antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The Biomedical Research Unit (BRU) of the University of KwaZulu-Natal provided Halothane and other accessories.

Grapefruit juice used was purchased from a local Woolworths Store, and it was declared to have the following nutritive contents: 190 kJ of energy, 0.6 g of protein, 10.0 g carbohydrate and 0.4 g of total dietary fibre. It had 0.0 g of both total fat and sodium. There were no

preservatives or additives used in the preparation of this grapefruit juice, according to the manufacturer‘s declaration.

2.2 Animal treatment and ethical clearance

Male Wistar Rats (*Rattusnovergicus*) of 200-300 g body weight (BW) provided by the Biomedical Research Unit were randomly assigned into five groups, (n=6). The animals were given free access to standard commercial chow and tap water *ad libitum*, and kept under maintained laboratory conditions of 25 ±2ºC temperature and 55% ±5% humidity. Water consumption by animals was assessed by measuring amounts of water left in drinking bottles at the end of each day. The animals were maintained at a 12-hour dark-to-light cycle, from 8:00 to 20:00 daily, and they were handled humanely, according to the guidelines prescribed by the Biomedical Research Ethics Committee of UKZN, which also granted ethical clearance/approval for this study (reference 106/13/Animal, see Appendix I).

2.2.1 STZ, GFJ and insulin treatment

Animals from Group 1, which were the controls, were treated with 3.0 ml/kg of body weight of distilled water by oral gavage. Similarly, Groups 3 and 4 were treated with 3.0 ml/kg body weight of GFJ. Diabetes was induced in groups 2, 4and 5by 60.0 mg/kg body weight single intraperitoneal injection of streptozotocin (STZ), dissolved in 0.2 mL of 0.1 M citrate buffer, pH 4.5 [98]. STZ treatment was performed after an overnight starvation of animals. Group 5 was further treated with regular insulin (4U/kg subcutaneously, twice a day). Three days after STZ was administered, blood glucose concentrations were measured by tail pricking, to confirm the development of diabetes. Rats with random blood glucose levels less than 11 mmol/L were considered non-diabetic, hence excluded from the study or re-induced [5, 7, 9].

On treatment day 60, animals were sacrificed by halothane overdose by placing them in an anaesthetic chamber with 100 mg/kg of halothane (Astra Zeneca Pharmaceuticals (Pty)

LTD,SA) for 3 minutes (each animal); and blood samples were collected by cardiac puncture. Plasma was frozen in a Bio Ultra freezer (Snijers Scientific, Holland, -80 °C) until used for different assays. Rat livers were excised, weighed, rinsed in normal saline and stored in liquid nitrogen (-180ºC) for further analysis of glycogen content and hepatic enzymes.

Table 2 Animal treatment protocol.GFJ (grapefruit juice); STZ (streptozotocin); D60 (STZ 60 mg/kg BW); GFJ-ND (GFJ-Non-diabetic); GFJ-D60 (GFJ + STZ 60 mg/kg BW); Ins-D60 (Insulin + STZ 60 mg/kg BW).

		Treatment				
Groups	**Designation**	**Distilled H_20** (ml, p.o).	**GFJ** (ml/kg, p.o.)	**Insulin** (U/kg SC)	**STZ** (mg/kg, SC)	**Glucose** (g/kg, IP)
1	Control	3.0				3.0
2	D60				60.0	3.0
3	GFJ-ND		3.0			3.0
4	GFJ-D60		3.0		60.0	3.0
5	Ins-D60			4.0	60.0	3.0

2.3 Experimental methods

2.3.1 Glucose monitoring

Fasting blood glucose (FBG) tests were performed on days 0 and 59 of treatment. The Glucose Tolerance Test (GTT) was done on treatment day 59. Droplets of blood obtained by means of tail pricking were mounted on glucose test strips and blood glucose analysed using a portable glucometer (Ascensia Elite™, Bayer Schering Pharma, Germany), using the glucose oxidase method. All treatment groups were starved overnight before the FBG tests

and GTT were performed. For the GTT, blood glucose concentrations were determined by tail pricking following intra-peritoneal injection of 3.0 g/kg body weight of glucose, in normal saline. Blood glucose levels were then monitored and analysed at time intervals of 0, 15, 30, 60 and 90 minutes, for all treatment groups. Area under the curve (AUC) was calculated from blood glucose-time curves and presented as AUC units (mM x minutes).

2.3.2 Plasma insulin

An ultra-sensitive rat insulin enzyme-linked immunoassay kit (DRG Diagnostics, Marburg, Germany) was used to analyse the plasma insulin levels, according to the manufacturer‘s manual/protocol. Samples of 25 µl were and 100 µl of enzyme conjugate were added on a 96-well plate, and incubated on a plate shaker (900 rmp) at 25 ºC for 2 hours. The plate was then washed with 700 µl of wash buffer. This was done 6 times. Thereafter, 200 µl of substrate tetramethylbenzidine (TMB) was added into each well and incubated at 25 ºC for 15 minutes. Stop solution (15 µl) was then added to each well and incubated for 5 minutes on a plate shaker, after which optical density was measured in a microplate reader (EZ Read 400, biochrom®) at 450 nm.

2.3.3 Hepatic glycogen

Hepatic glycogen content was measured by the modified method of Seifter *et al.,* [99]. The liver tissues were homogenised using 5 volumes of 4 M KOH (ice cold). The homogenate was then dissolved by boiling in a water bath (100° C) for 30 minutes. The glycogen was then precipitated with ethanol, thereafter pelleted, washed, and solubilized in distilled water. Treatment with anthrone reagent followed to assay the glycogen concentration of the livers (92 g/L anthrone in 95% (v/v) H_2SO_4), and the absorbance measured at 620 nm. Glycogen content was expressed as mg/g liver protein.

2.3.4 Hepatic enzymes

Glucokinase activity

Glucokinase activity in the livers of different treatment groups was determined by spectrophotometric methods that had been previously described by Davidson and Arion [100] and Barzila and Rosetti [101]. Liquid nitrogen frozen livers were aliqouted into tubes (100 mg/ tube) and homogenated in buffer containing: 50 mM HEPES, 100 Mm KCl, 2.5 mMdithiothreitol, 1 mM EDTA and 5 mM $MgCl_2$. The resulting homogenates were then centrifuged at 100,000 g for 60 minutes at 4^{o}C. The supernatant was used for spectrophotometric measurements, while the microsomal residue was kept for use in the glucose-6-phosphatase assay. The Bradford assay [102] was performed to determine protein concentrations. Glucose-6-phosphatase formation from glucose was coupled to oxidation by glucose-6-phosphate dehydrogenase and NAD^+ in a reaction mix containing the following: 50 mM HEPES, 100 mMKCl, 2.5 mMdithiothreitpl, 7.5 mM $MgCl_2$, 5 mM ATP, 10 mg/ml albumin and glucose of six different concentrations (0, 5, 10, 15, 25, 50 mM, respectively). Also in the reaction mix were 0.5 mM NAD^+, 4 units of glucose-6-phosphate dehydrogenase, from a fermentation associated bacteria (*Leuconosticmesenteroides*), and 1 mg (wet weight) of liver. ATP was added to the mixture to initiate the reaction and NAD^+ reaction rate was recorded at 340 nm for half an hour, at the tempearature of 37^{o}C. A Beckman DU-70 spectrophotometer with an internal temperature controller was used. Enzyme activity was expressed as μmol/g liver protein protein/minute.

Glcose-6-phosphatase (G6Pase) activity

Liver samples (100 mg) that were previously frozen in liquid nitrogen were aliqouted into tubes. Buffer containing the 50 mM-HEPES, 100 mM-KCl, 2.5mM-dithiothreitol, 1 mM-EDTA and 5 mM-$MgCl_2$ was added to the tubes to homogenise the tissues. The homogenates

were then centrifuged at the speed of 100, 000 x g for 60 minutes, at 4^{o}C, to get the residual microsomal fraction. Glucose-6-phosphatase liver content by the modified spectrophotometric method of Lange *et al*., [103]. Total content protein was determined by the Bradford method [102]. The microsomal fraction was then incubated in three different concentrations of glucose-6-phosphate (0, 0.5, 1.0, 2.5, 5 and 10 mM). This reaction was carried out at 37^{o}C and after 30 minutes stopped with a solution containing the following reagents: acid molybdate, with 2/9 volumes of 10% SDS, and 1/9 volume of 10% ascorbic acid. This mix was then incubated at 45^{o}C for 20 minutes, and the absorbance recorded at 820 nm. A Beckman DU-70 spectrophotometer with a temperature controller was used to measure the absorbance. The basis of this assay was the hydrolysis of glucose-6-phosphate by the microsomal fraction containing glucose-6-phosphatase. A standard curve was drawn using the different concentrations of free phosphate, and glucose-6-phosphatase enzyme activity expressed in µmol/min/g of liver protein.

Phosphoenolpyruvate carboxykinase (PEPCK) activity

PEPCK activity was determined as per the modified methods of Bentle and Lardy [104] and Stiffin *et al.* [105]. Cytosolic fraction was obtained from homogenised liver tissues by centrifugation at 100,000 g for 1.0 hr at 4°C, and the activities of enzymes measured in a final reaction volume of 1.0 ml, at pH 7.0, containing 50 mM-sodium HEPES/KOH buffer, 10.0 mM-IDP, 1.0 mM-$MgCl_2$, 1.0 mM-dithiothreitol, 0.25 mM-NADH, 2.0 mM-hosphoenolpyruvate, 50 mM-Na_2CO_3 and 10 U of malic dehydrogenase (1 unit defined as 1 µmol of malate produced/min/mg of liver protein). Total protein content was determined by Bradford method [102]. All assay components were pre-incubated for 3 minutes. The enzyme activity was measured at 25°C and 340 nm, using Beckman DU-70 spectrophotometer equipped with temperature controller, and expressed as mmol of oxaloacetate (OAA) formed/min/g of liver protein.

2.3.5 Plasma lipids

An automated chemistry analyser (Labmax 240) was used to analyse fasting total cholesterol, HDL cholesterol and triglycerides, using commercial kits purchased from ACE Chemicals Ltd. (Seoul, South Korea). The HDL fractions were separated by the heparin-manganese procedure as previously described [106], while the LDL cholesterol was calculated according to the Friedwald formula: LDL = total cholesterol – (HDL cholesterol + VLDL cholesterol [107]. VLDL was calculated as VLDL = triglycerides/5 [108]. The atherogenic index was calculated as the: LDL cholesterol to HDL cholesterol ratio (LDL: HDL).

2.3.6 Western blot analysis: ACAT and HMG-CoA reductase

Protein Extraction

Liquid nitrogen frozen hearts and livers were homogenised in ice-cold Tris-sucrose buffer (100 mmol/L Tris, 250 mmol/L sucrose at pH 7.6), using a motor driven Teflon glass homogeniser at 504 x g, in the presence of complete protease inhibitor cocktail. The homogenate was then ultra-centrifuged, at 100 000 x g for 60 minutes, at 4ºC. The pellet was then re-suspended in HEPES-sucrose buffer (10 mmol/L HEPES, 300 mmol/L sucrose, pH 7.5, protease inhibitor). Total protein content was then determined using the Bradford Assay [102].

Western Blot Analysis

Protein samples (50µg) were separated using 12.5% SDS–PAGE for 60 minutes at 120 V in a tank buffer (Electrode Buffer – X1), using the Mini-Protean 3 cell (Bio Rad) system. The PageRuler^{TM}prestained protein ladder (Fermentas, Thermo Scientific) was used as a molecular weight marker. The PAGE gel was then transferred onto a nitrocellulose membrane (Amersham^{TM}Hybond^{TM}nitrocellulose membrane, GE Healthcare) at room temperature for 60 minutes, at 100 V, in a transfer buffer, with cooling. The nitrocellulose membranes were then blocked for one hour using 6% (w/v) skim milk in Tris-buffered saline with tween for 60 minutes at room temperature, with agitation, to block non-specific binding of the primary antibody. This nitrocellulose membrane, after being rinsed twice with Tris-buffered saline, was then incubated overnight at 4ºC with the primary antibody overnight. The nitrocellulose membrane was then rinsed six times for ten minutes each to remove non-specifically bound primary antibody. The nitrocellulose membrane was then probed with horseradish peroxidise labelled secondary antibodies, at room temperature for an hour. The membrane was then washed in Tris-buffered saline with Tween, six times for ten minutes

each, to remove any unbound secondary antibodies. The nitrocellulose membrane was then visualised using the Immn-Star^{TM}HRPChemiluminescent kit (Bio Rad) on the ChemiDoc^{TM} XRS system, with Image Lab^{TM}software (Bio Rad).

The primary antibodies used were HMGCR (C-1) mouse monoclonal antibody (to be diluted 1:1000), ACAT-2 (N-15) goat polyclonal antibody (to be diluted 1:1000) and β-actin as the reference protein (to be diluted 1:2000). The secondary antibodies used were horseradish peroxidise-goat anti mouse and horseradish peroxidase-conjugate mouse anti-goat antibodies for the HMGCR and ACAT-2, respectively.

2.3.7 Statistical analysis

The data are presented as mean ± standard deviation and analysed by the One-way ANOVA or the non-parametric Mann-Whitney tests and/or student t-tests were applied to the results to determine statistical significance, using Graph pad Prism Software Version 5.0. (Graphpad Prism® Software, Inc. San Diego, CA). Values of $p < 0.05$ were taken to imply statistical significance.

CHAPTER 3

Results

3.1 Weights and water Consumption

In comparison to the controls, diabetic rats had significantly ($p<0.05$) reduced weights. Treatment of diabetic with insulin or with grapefruit juice significantly ($p<0.05$) improved the weights of the diabetic animals (Figure 4). There was a significant ($p<0.05$) increase in average water consumption among the diabetic groups in comparison to the control. Treatment with grapefruit juice significantly ($p<0.05$) reduced or improved water intake among the diabetic animals (Figure 5).

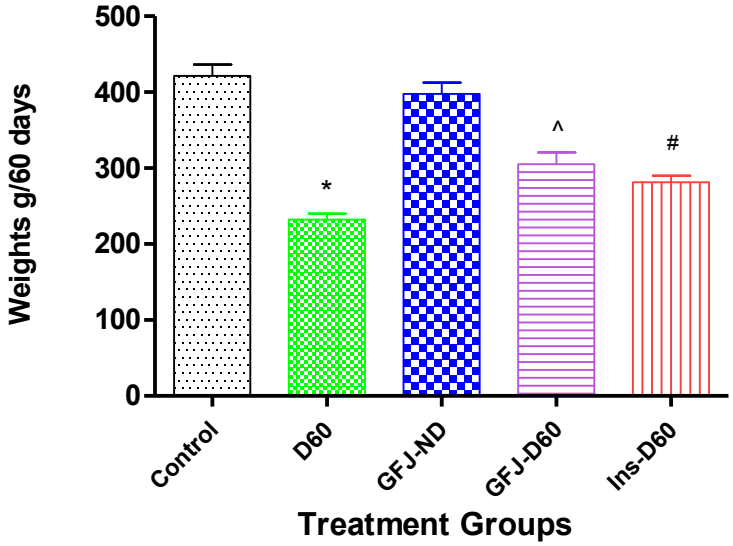


Figure 4: Total body weight differences in animals before and after treatment. * $P<0.05$ compared to control, ^ $P<0.05$ compared to D60, # $P<0.05$ compared to D60. Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60, grapefruit juice and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg.

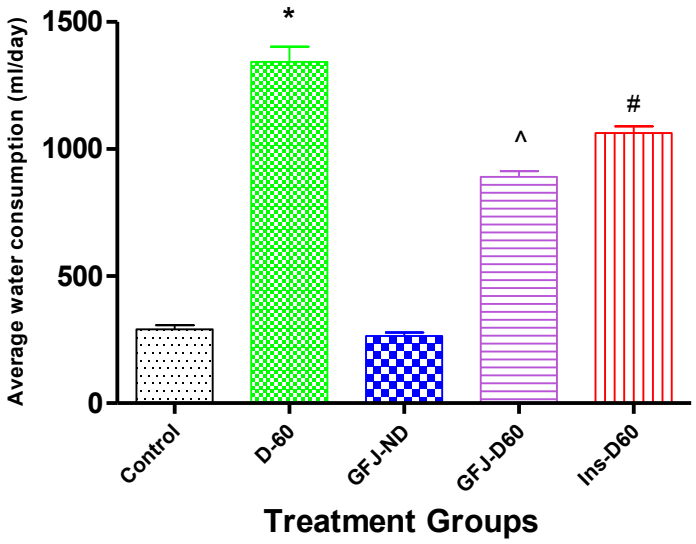


Figure 5: Average daily water consumption by animals over a 60 day treatment. * P<0.05 compared to the control, ^ P<0.05 compared to D60, # P<0.05 compared to D60. Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60, grapefruit juice and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg.

3.2 Glucose monitoring

Fasting blood glucose concentrations were significantly ($p<0.05$) increased in diabetic animals in comparison to the controls. Grapefruit juice treatment, resulted in a significant ($p<0.05$) reduction in fasting plasma glucose levels compared to the diabetic non-treated group (Figure 6). Diabetic rats displayed impaired glucose tolerance, while treatment with grapefruit juice significantly ($p<0.05$) improved the observed glucose intolerance (Figure 7).

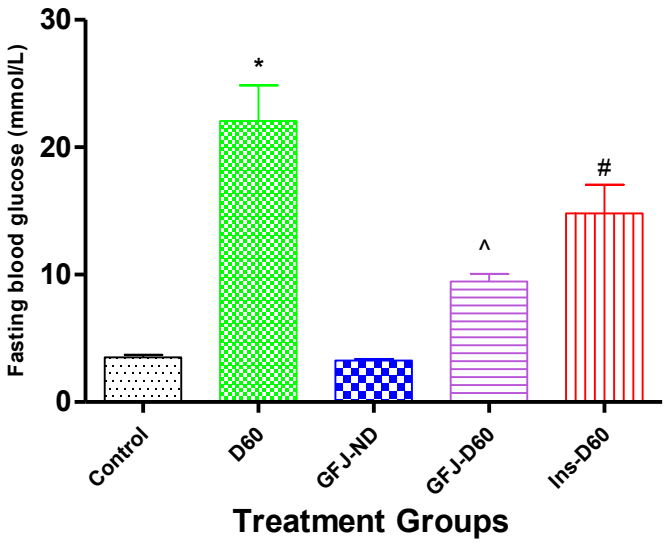
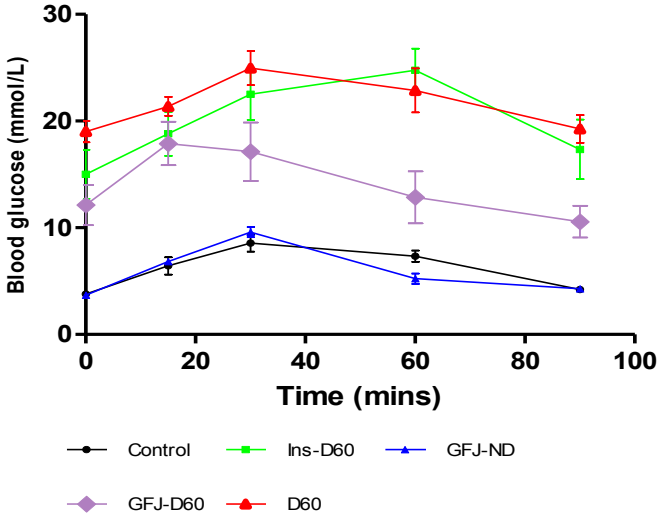


Figure 6: Concentrations of fasting blood glucose of animals on the 59th day of treatment. No insulin was administered before test. * P<0.05 compared to the control, ^ P<0.05 compared to D60, # P<0.05 compared to D60. Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60, grapefruit juice and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg.

A.



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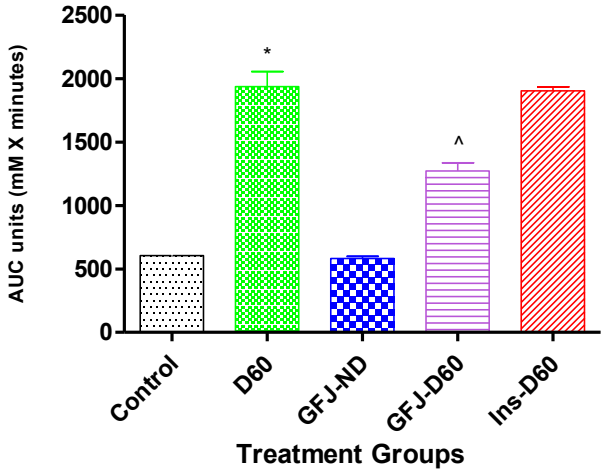


Figure 7: Glucose Tolerance Tests after 0, 15, 30, 60 and 90 minute intervals of treatment (A), and AUCs calculated as explained in the methods (B). *P<0.05 compared to control; ^P<0.05 compared to D60.Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60, grapefruit juice and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg.

3.4 Plasma insulin

Controls and non-diabetic grapefruit juice treated animals exhibited similar fasting plasma insulin levels, which were, however, significantly ($p<0.05$) reduced in diabetic animals. Diabetic insulin (Ins-D60) treated animals had significantly ($p<0.05$) higher fasting plasma insulin levels in comparison to diabetic grapefruit (GFJ-D60) and diabetic control (D-60) animals, respectively. There was no significant elevation in fasting plasma insulin levels observed in diabetic GFJ treated rats compared to the diabetic ones (Figure 8).

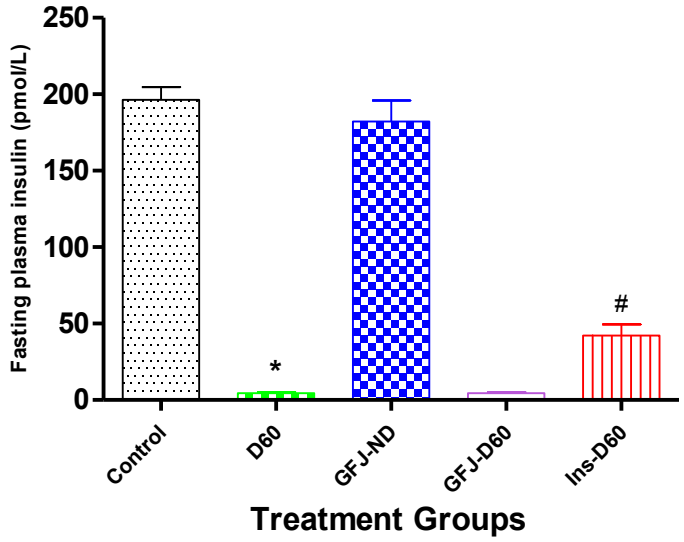


Figure 8: Fasting plasma insulin. Insulin treatment was withheld on test day. *P<0.05 compared to the control, #P<0.05 compared to D60. Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60grapefruit juice and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg.

3.5 Hepatic glycogen

Diabetic non-treated animals (D60) appeared to have reduced glycogen content compared to the controls (Figure 9). Diabetic animals treated with GFJ had significantly elevated ($p <0.05$) glycogen content levels in comparison to diabetic non-treated animals (D60).

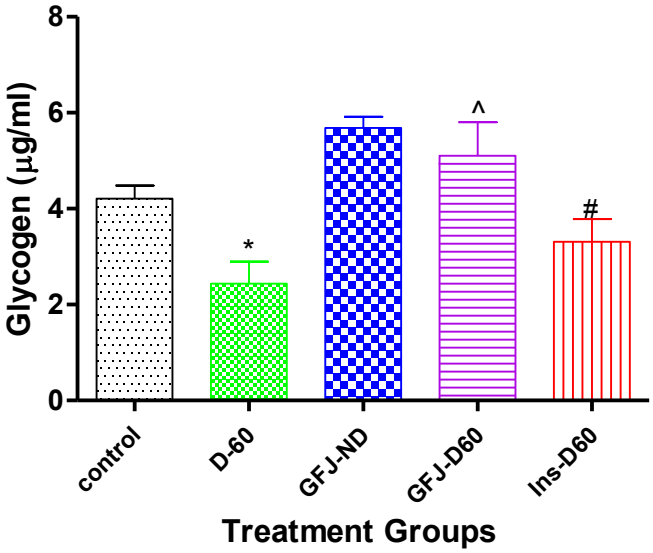


Figure 9:Liver glycogen content concentrations of the different treatment groups. * P<0.05 compared to control, ^P<0.05 compared to D60, # P<0.05 compared to GFJ-D60. Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60, grapefruit juice and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg.

3.6 Glucokinase activity

When plotted against substrate concentrations, hepatic glucokinase activity obeyed The Michaelis-Menten kinetics (Figure 10). Linear regression analysis of Eadie-Hofstee plots [100], (Table 3) showed a significant ($p<0.05$) reduction in glucokinase activity in diabetic rats (Vmax, 13.4 (D60) µmol/g of liver tissue/ min); when compared to the control (Vmax; 16.8 µmol/g of liver tissue/ min)rats. In comparison to non-treated rats, treatment with GFJ alone, {Vmax; 14.5µmol/g of liver tissue/ min (GFJ-D60); insulin alone {Vmax; 20.3 µmol/g of liver tissue/ min (Ins-D60); significantly increased glucokinase activities ($p<0.05$). GFJ treatment resulted in a similarly significant ($p<0.05$) increaseinglucokinase activity in non-diabetic animals compared to controls.

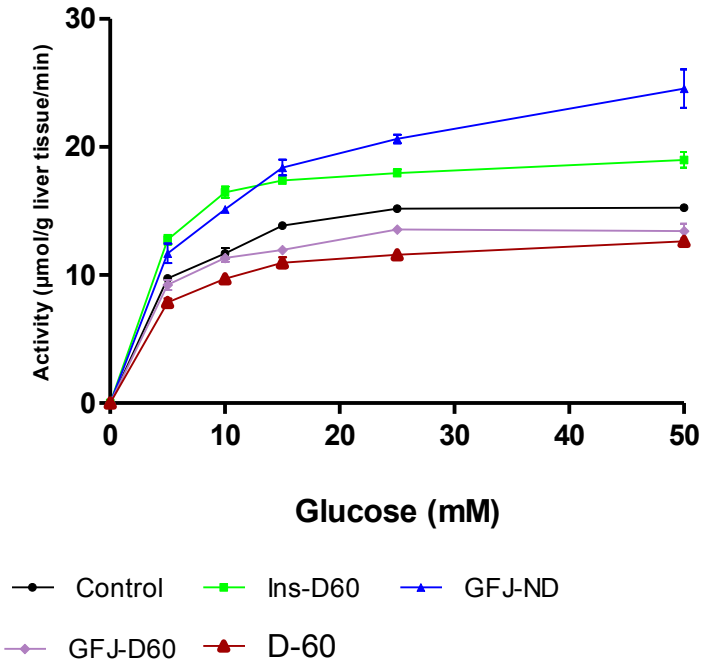


Figure 10: Michaelis-Menten kinetics of glucokinase activity in the livers of treated rats. Glucokinase activity was plotted against substrate concentrations. Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60, grapefruit juice and

diabetic, 60 mg/kg; GFJ-Ins-D60, grapefruit juice and insulin combination, and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg.

Table 3 Calculated glucokinase activity as determined by Eadie-Hofstee plots. Statistically significant differences are marked by relevant symbols applicable to different treatment groups. Vmax = (μmol/g of liver tissue/min). * P<0.05 compared to D-60; λ P<0.05 compared to GFJ-ND; + P<0.05 compared to Ins-D60; ^P<0.05 compared to GFJ-D60.

	Control	**D60**	**GFJ-ND**	**GFJ-D60**	**Ins-D60**
	Mean + SD	Mean + SD	Mean + SD	Mean + SD	Mean + SD
V_{max}	16.8 +/- 0.4	13.4 +/- 0.3	23.0 +/- 0.9	14.5 +/- 0.3	20.3 +/- 0.4
K_m (mM)	0.27	0.281	0.21	0.35	0.37
r^2	0.9107	0.9276	0.8971	0.8703	0.8937
V_{max}/ K_m	4.5	3.7	4.9	5.1	7.4
P<0.05	*, λ	*,+,^	λ	^	+

3.7 Glucose-6-phosphatase activity

Glucose-6-phosphatase activity, similarly obeyed Michaelis-Menten kinetics when plotted against substrate concentrations (Figure 11). The linear regression of data transformed inEadie-Hofstee [100] plots (Table 4) showed that Glucose-6-phosphotase activities were significantly ($p< 0.05$) increased in diabetic {Vmax: 22.6 (D60) µmol/min/g liver tissue; Vmax/Km:102.0 (D60) in comparison to the controls (Vmax: 12.5 µmol/min/g liver tissue; Vmax/Km:53.39) (Table 3). Subsequent treatment with either GFJ alone {Vmax; 18.8 (D60) µmol/min/g liver tissue; Vmax/Km: 96.56 (D60)} or insulin alone (Vmax: 18.0 µmol/min/g liver tissue, Vmax/Km: 56.62), resulted in a significant ($p<0.05$) decrease in Glucose-6-phosphatase activity diabetic compared to non-treated diabetic D60, animals (Table 3). Glucose-6-phosphatase activity in GFJ-treated non-diabetic rats (Vmax: 11.8µmol/min/g liver tissue; Vmax/Km: 48.48) was similar to that of the controls.

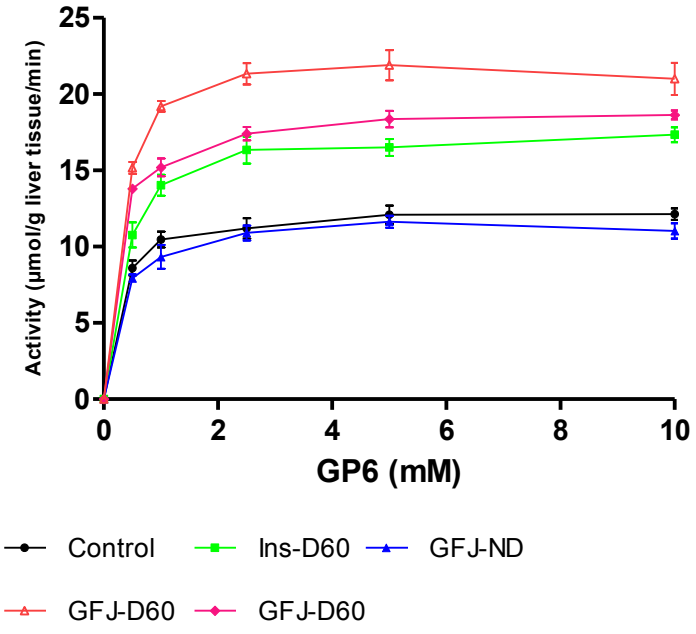


Figure 11:Michaelis-Menten kinetics of glucose-6-phosphatase activity in the livers of rats treated *in vivo*. Glucose-6-phosphatase activity was plotted against substrate concentrations.

Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60, grapefruit juice and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg.

Table 4 Calculated G6Pase activity as determined by Eadie-Hofstee plots. Statistically significant differences are marked by relevant symbols applicable to different treatment groups. Vmax = (µmol/g of liver tissue/min). *P<0.05 compared to D60; +P<0.05 compared to Ins-D60; ^P<0.05 compared to GFJ-D60.

	Control	**D60**	**GFJ-ND**	**GFJ-D60**	**Ins-D60**
	Mean + SD	Mean + SD	Mean + SD	Mean + SD	Mean + SD
V_{max}	12.5 +/- 0.3	22.6 +/- 0.4	11.8 +/- 0.3	18.8 +/- 0.3	18.0 +/- 0.5
K_m (mM)	4.61	4.520	4.12	5.13	3.15
r^2	0.7253	0.7577	0.7356	0.8702	0.8265
$V_{max/}$ K_m	57.39	102.0	48.48	96.56	56.62
P<0.05	*	*, +, ^		^	+

3.8 Phosphoenolpyruvate carboxykinase (PEPCK) activity

Similarly PEPCK activity Michaelis-Menten kinetics when plotted against substrate concentrations (Figure 12). Linear regression analysis of Eadie-Hofstee plots [100], (Table 5) showed significantly ($p<0.05$) increased PEPCK activity in diabetic animals [Vmax: 22.6±0.6 (D60) µmol/min/g liver tissue; Vmax/Km: 102.0 (D60)] compared to controls (Vmax: 12.5 +/- 0.3;Vmax/Km: 57.39). Treatment of diabetic rats with GFJ [Vmax: 18.8 +/-0.3 (GFJ-D60) µmol/min/g liver tissue; Vmax/Km: 96.56 (GFJ-D60)] significantly ($p<0.05$) reduced PEPCK activity in diabetic rats. Similarly, treatment with insulin (Vmax: 18.0 +/- 0.5 µmol/min/g liver tissue, Vmax/Km: 56.62) significantly ($p<0.05$) reduced PEPCK activity in diabetic rats compared to non-treated diabetic rats (Table 5).

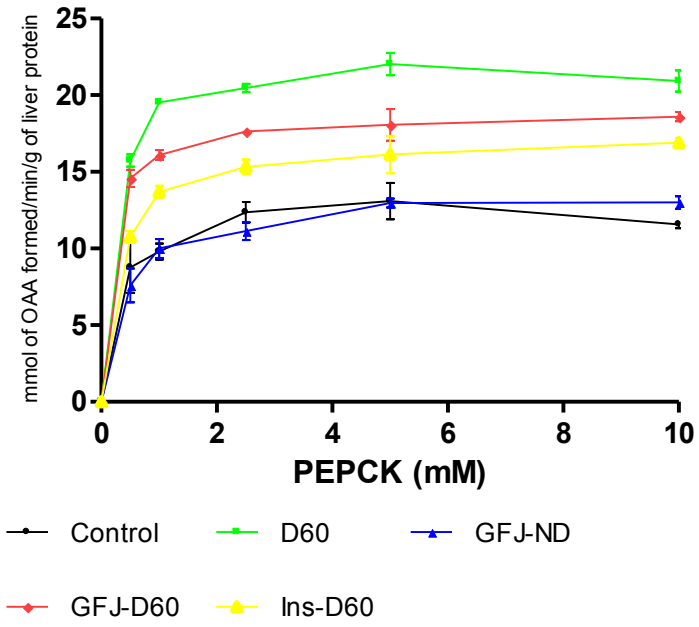


Figure 12: Michaelis-Menten kinetics of phosphoenolpyruvatecarboxykinaseactivity. PEPCK was plotted against OAA concentrations formed. D60, diabetic, 60 mg/kg; Ins-D60,

insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60, grapefruit juice and diabetic, 60 mg/kg.

Table 5: Calculated PEPCK activity as determined by Eadie-Hofstee plots. Statistically significant differences are marked by relevant symbols applicable to different treatment groups. Vmax = (µmol/g of liver tissue/min). *P<0.05 compared to D60; +P<0.05 compared to Ins-D60; ^P<0.05 compared to GFJ-D60.

	Control	**D60**	**GFJ-ND**	**GFJ-D60**	**Ins-D60**
	Mean + SD	Mean + SD	Mean + SD	Mean + SD	Mean + SD
V_{max}	12.5 +/- 0.3	22.6 +/- 0.6	11.8 +/- 0.3	18.8 +/- 0.3	18.0 +/- 0.5
K_m (mM)	4.61	4.520	4.12	5.13	3.15
r^2	0.7253	0.7577	0.7356	0.8702	0.8265
V_{max}/ K_m	57.39	102.0	48.48	96.56	56.62
P<0.05	*	*, +, ^		^	+

3.9 Plasma lipid profiles

In comparison to the non-treated diabetic group (D60), the total plasma cholesterol levels were significantly ($p<0.05$) higher in the grapefruit juice treated diabetic group (Figure 13). HDL-cholesterol levels were also higher in the diabetic grapefruit juice treated group versus the diabetic non treated group ($p<0.05$) (Figure 14). Conversely, LDL-cholesterol levels were significantly higher in the diabetic non treated group in comparison to the control group ($p<0.05$) and the diabetic insulin treated group ($p<0.05$) (Figure 15).

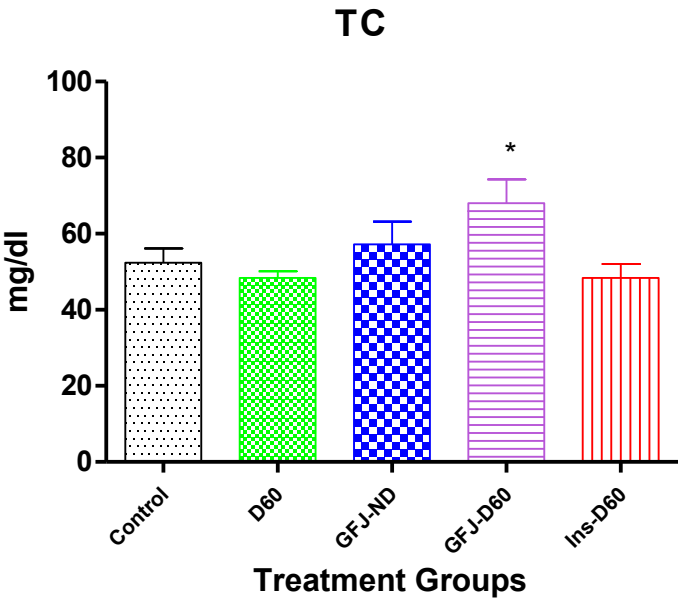


Figure 13: Total cholesterol (TC) levels of different treatment groups. *P<0.05 compared to D60. Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60 grapefruit juice and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg.

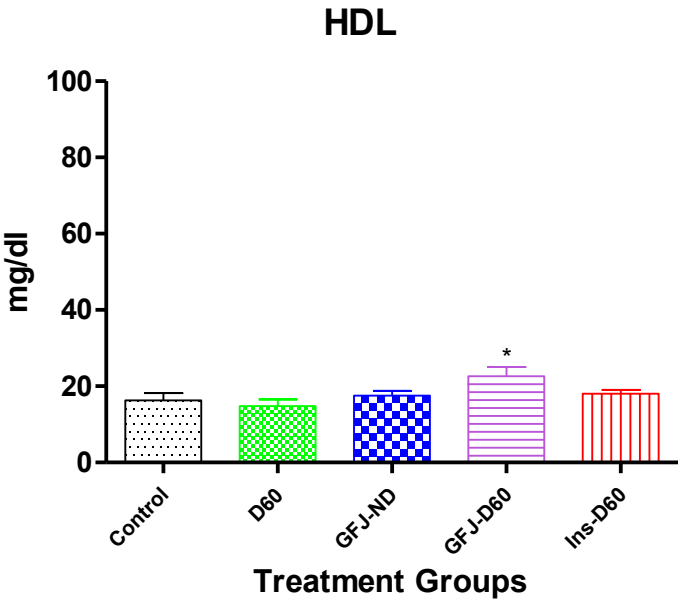


Figure 14: HDL-cholesterol levels of the different treatment groups. *P<0.05 compared to D60. Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60 grapefruit juice and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg

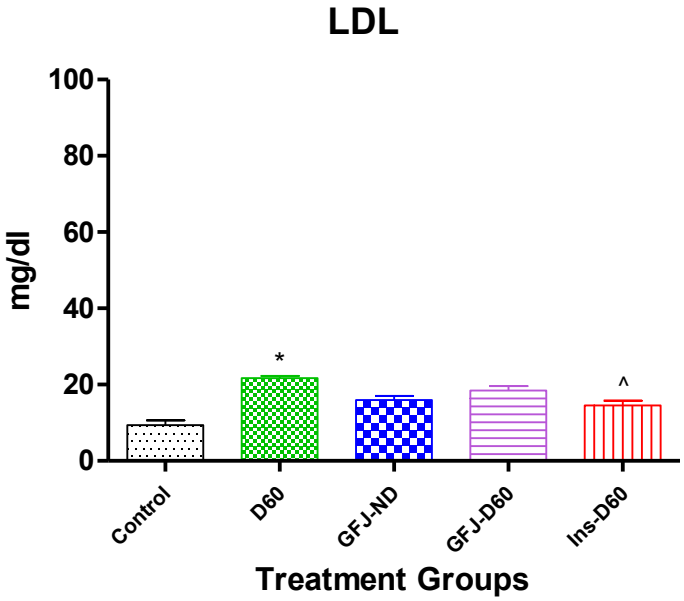


Figure 15: LDL-cholesterol levels of the different treatment groups. LDL calculated as LDL = total cholesterol – (HDL cholesterol + VLDL cholesterol). * P<0.05 compared to the control, ^ P<0.05 compared to D60. Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60 grapefruit juice and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg.

3.8 Atherogenic index

The atherogenic index, calculated as LDL to HDL ratios (LDL:HDL) was significantly ($p<0.05$) higher in the non-treated diabetic compared to the control group (Figure 16). The grapefruit juice treated diabetic and the insulin treated diabetic groups had significantly ($p<0.05$, respectively) reduced atherogenic indexes compared to the non-treated diabetic group (Figure 16).

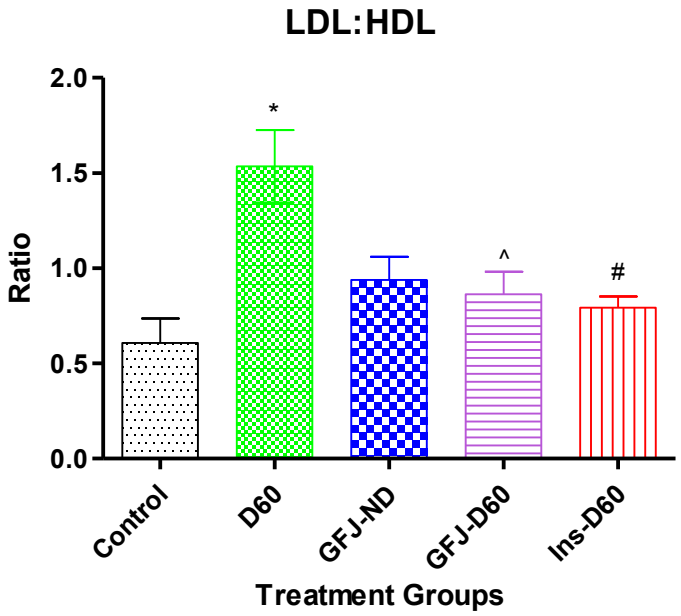


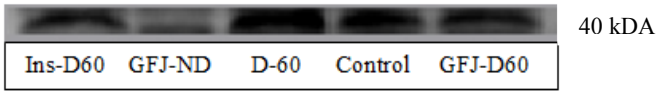
Figure 16: Atherogenic Index (LDL:HDL ratio) of the different treatment groups. * P<0.05 compared to the control, ^ P<0.05 compared to D60, # P<0.05 compared to D60. Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60 grapefruit juice and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg.

3.8 Hepatic cholesterol synthesis: ACAT and HMG-CoA reductase

Animals from the non-treated diabetic group had significantly elevated ACAT enzyme expression in rat livers compared to the control (Figure 17A and 17B). Grapefruit juice-treated diabetic animals (GFJ-D60) had significantly ($p<0.05$) reduced ACAT enzyme expression in comparison to non-treated diabetic animals (Figure 17A and 17B). Similarly, animals from the insulin-treated diabetic group had significantly reduced liver ACAT enzyme expressions (Figure 17A and 17B). Animals from the non-treated diabetic group had significantly ($p<0.05$) elevated hepatic HMG-CoA reductase enzyme expression in compared to the control group. Grapefruit juice-treated diabetic animals (GFJ-D60) had significantly

(p<0.05) reduced hepatic HMG-CoA reductase enzyme expression compared to the non-treated diabetic group (Figure 18A and 18B).

A)



B)

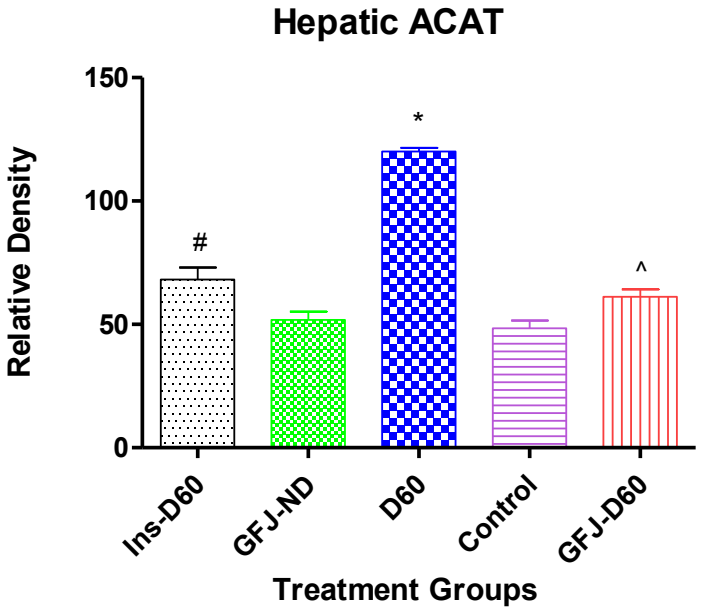
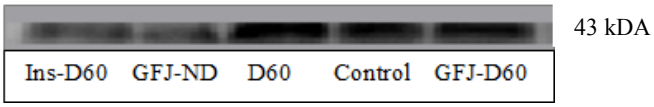


Figure 17A): Immuno-reactive bands reflecting the liver enzyme ACAT in liver tissues of rats. Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60, grapefruit juice and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg, n=6.**B)** Hepatic ACAT expression relative densities for the different treatment groups. # P<0.05 compared to D60, * P<0.05 compared to the control, ^ P<0.05 compared to D60. Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60, grapefruit juice and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg.

A)



B)

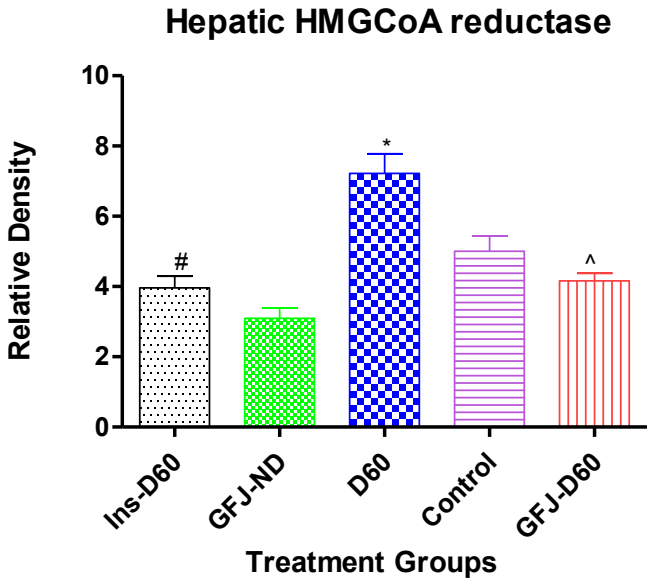


Figure 18A): Immuno-reactive bands reflecting the liver enzyme HMG-CoA reductase in the liver tissues of rats. Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60, grapefruit juice and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg, n=6. **B)**: Hepatic HMG-CoAreductase expression relative densities for the different treatment groups.# P<0.05 compared to D60, * P<0.05 compared to the control, ^ P<0.05 compared to D60. Ins-D60, insulin and diabetic, 60 mg/kg; GFJ-ND, grapefruit juice and non-diabetic; GFJ-D60, grapefruit juice and diabetic, 60 mg/kg; D60, diabetic, 60 mg/kg.

CHAPTER 4

Discussion

This study was aimed at investigating the anti-diabetic and anti-dyslipidaemic effects of grapefruit juice in type 1 STZ-induced diabetic rats. The findings suggest that GFJ does have glucose lowering and lipid profile improving effects on diabetic rat models. Although grapefruit juice treatment did not result in significant LDL-C level changes, the anti-dyslipidaemic effects of grapefruit juice were suggested by the down regulation of hepatic enzymes ACAT and HMG-CoA reductase. These results are significant in that they highlight and contextualise GFJ and/or its bioactive chemical constituents as having hypoglycaemic and anti-dyslipidaemic effects.

4.1 Hypoglycaemic effects of Grapefruit Juice

Treatment of animals with 60 mg/kg body weight of STZ appears to have resulted in the destruction of beta cells, leading to the induction of type 1, insulin dependent diabetes in the animals. The diabetic animals had significantly ($p<0.05$) reduced weight gain (or increased weight loss) during normal growth (compared to the control), suggesting accelerated lipid and protein breakdown in the absence of insulin (D60). Treatment with grapefruit juice and/or insulin resulted in a significant ($p<0.05$) improvement in weight loss among the diabetic animals (Figure 4). This suggests that treatment with grapefruit juice or insulin has the ability to prevent and reduce proteolysis, as well as lipolysis; which are associated with the deficiency of insulin. However, these treatments do not affect natural growth in non-diabetic animals.

Diabetic animals had significantly ($p<0.05$) higher water consumption in comparison to the controls, indicating polydipsia. Treatment with grapefruit juice or with insulin, significantly

($p<0.05$) reduced water consumption in diabetic animals (Figure 5). The reduction in water consumption demonstrated by diabetic animals treated with grapefruit juice suggests that grapefruit juice has polydipsia and polyuria reducing effects.

Fasting blood glucose levels of STZ-induced diabetic rats were significantly ($p<0.05$) higher than those of the controls (Figure 6), suggesting loss of beta cells functionality and the absence of insulin after treatment with 60 mg of STZ. STZ [2-deoxy-2(3-methyl-3-nitrosoureido)-D-glucopyranose] is a broad spectrum antibiotic that was initially extracted from *Streptomyces acromogens* [98, 109, 110], which was first reported to have diabetic effects in rats, mice and other mammals back in 1963 [98, 109, 110]. STZ is thought to exert its diabetic effects by the degranulation of β-cells through reactive oxygen species (ROS) [109]. The results therefore suggest that type 1 diabetic models were successfully created.

Treatment of diabetic animals with insulin (Ins-D60) significantly ($p<0.05$) reduced fasting blood glucose levels, compared to non-treated animals (D60). Treatment with grapefruit juice (GFJ-D60) similarly significantly ($p<0.05$) reduced fasting blood glucose levels in comparison to non-treated diabetic rats (D60). This indicates that GFJ has hypoglycaemic effects similar to those of insulin. Diabetic rats exhibited impaired glucose tolerance in GTT (Figure 7A) and calculated AUCs (Figure 7B) showed that treatment with significantly ($p<0.05$) improved glucose intolerance in diabetic rats. Hypoglycaemic effects of grapefruit juice in non-diabetic animals has been reported [84]. However, the effects of grapefruit juice in conditions of hyperglycaemia have not previously been shown. Although diabetics widely consume grapefruit juice in hope for improve diabetes, this study suggests that grapefruit juice treatment has anti-diabetic effects.

4.2 Effects of grapefruit juice on plasma insulin levels

Diabetic rats (Ins-D60, GFJ-D, and D60) had significantly reduced plasma insulin levels in comparison to non-diabetic rats (Figure 8). The significantly reduced plasma insulin levels of STZ-induced diabetic rats can be attributed to the destruction of β-cells by STZ, and hence reduced plasma insulin levels [109]. Treatment with grapefruit juice of diabetic animals (GFJ-D) did not significantly increase plasma insulin levels in comparison to non-treated diabetic animals (Figure 8); neither did treatment with GFJ of non-diabetic rats affect plasma insulin levels, in comparison to the controls. This suggests that grapefruit juice and/or its bioactive compounds do not enhance insulin production/secretion, nor does it repair damaged pancreatic β-cells. The non-insulinotropic effects of grapefruit juice have been previously reported [84].

4.3 Effects of grapefruit juice on hepatic glycogen content

Non-treated diabetic animals (D60) had significantly ($p<0.05$) reduced liver glycogen levels compared to the controls (Figure 9). Treatment of diabetic and non-diabetic rats with grapefruit juice (GFJ-D60 and GFJ-ND) significantly increased glycogen levels compared non-treated diabetic and the control group rats, respectively (Figure 9). This suggests that treatment with grapefruit juice promotes glycogen synthesis. GFJ could possibly achieve this by suppressing insulin counter-regulating hormones. Alternatively, grapefruit juice treatment could also exert glycogen synthesis by activating the mitogen activated protein kinase signalling pathways, resulting in the phosphorylation of protein phosphatase-1, which activates glycogen synthase; leading to the synthesis of glycogen.

4.4 Grapefruit juice effects on glucokinase, G6Pase and PEPCK activity

Glucokinase is an enzyme responsible for the conversion of glucose into glucose-6-phosphate [7]. In this study, glucokinase activity was significantly reduced in diabetic non-treated (D60) animals in comparison to the controls (Figure 10) and (Table 3). Grapefruit juice treatment

and insulin treatment significantly ($p<0.05$) improved glucokinase activity of the animals, in comparison to diabetic non-treated animals (Table 3). Furthermore, the treatment of non-diabetic rats with grapefruit juice significantly ($p<0.05$) compared to the controls; and far more than the insulin treated diabetic animals.

G6Pase functions by regulating the rate-limiting step in gluconeogenesis (the hydrolysis of glucose-6-phosphate from glycogen) [111], thus contributing to elevated glucose levels. In this study, G6Pase activity was significantly reduced by GFJ treatment. Jung *et al*., [112] have reported that the bio-active components of grapefruit juice, naringin and hesperidin reduce blood glucose levels in mice by elevating glucokinase and glycogen concentrations, and concomitantly suppressing G6Pase.

PEPCK regulates the rate-limiting steps in hepatic gluconeogenic flux by converting oxaloacetate to phosphoenolpyruvate and carbon dioxide, respectively [111]. Diabetic animals displayed significantly ($p<0.05$) increased PEPCK activity compared to the controls (Figure 12 and Table 5). Treatment with grapefruit juice as well as with insulin significantly ($p<0.05$) reduced PEPCK enzyme activity in diabetic animals compared to the non-treated diabetic animals.

The study did not demonstrate how grapefruit juice increases the activity of glycolytic glucokinase, resulting in increased hepatic synthesis, nor did it demonstrate whether grapefruit juice suppresses gluconeogenic enzymes by either increasing cellular protein content or gene transcription. However, considering that GFJ treatment was chronic in these animals, and the similarity between GFJ and insulin effects, the enhancement of gene expression of these cannot be ruled out. The effects of GFJ juice seem to be enhanced by insulin, and it is tempting to speculate that GFJ, could, like metformin, be up regulating the ~~–m~~aster energy sensor" (AMPK) which in turn could be activating glucokinase and simultaneously deactivating G6Pase and PEPCK, respectively through phosphorylation in the

short-term or increased gene expression in the long-term. While the anti-diabetic effects of flavonoids are well documented [95], grapefruit juice as opposed to pure forms of isolated flavonoids was chosen for this study. This was on the basis of evidence suggesting naringin does not exert hypoglycaemic effects in type 1 diabetic model [3], suggesting that insulin is required for hypoglycaemic effects of flavonoids and by extension GFJ as demonstrated in the present study.

4.5 Plasma lipids profiles

The key goal of treating dyslipidaemia is focused on reducing LDL-cholesterol and TG levels, as well as increasing HDL-cholesterol levels [28, 113]. Non-treated diabetic rats had significantly elevated LDL-cholesterol levels compared to the controls (Figure 14), suggesting diabetic dyslipidaemia. Grapefruit juice treatment of diabetic rats (GFJ-D60) significantly ($p<0.05$) increased total cholesterol levels as well as HDL ($p<0.05$) levels, in comparison to non-treated diabetic rats (D60) (Figures 12 and 13 respectively). The observed increase in total cholesterol levels by grapefruit juice treatment can thus be attributed to the increase in HDL-cholesterol fraction. Atherogenic index, expressed as LDL:HDL ratio, was significantly ($p<0.05$) higher in non-treated diabetic rats in comparison to the controls. The effects of grapefruit juice treatment of diabetic animals seemed to be similar to those exerted by insulin in improving the atherogenic index (Figures15). This study suggests that treatment with grapefruit juice has anti-dyslipidaemic effects.

4.6 Hepatic cholesterol synthesis

The results of our study show that ACAT and HMG-CoA reductase protein expression were significantly elevated in non-treated diabetic animals (D60) in comparison to the diabetic grapefruit juice-treated animals (GFJ-D60) as well as the controls (Figure 17a and 17b). ACAT is an important cholesterol-regulating enzyme that is involved in cholesterol esterification and absorption [3, 63, 114]. ACAT is also known to be involved in the secretion

of LDL-cholesterol, as well as the accumulation of the fatty material in the arterial wall, which is one of the important metabolic changes in the progression of atherosclerosis [63]. The down regulation of ACAT protein expression by grapefruit juice treatment of diabetic rat models resembles that of known ACAT inhibitors [63], suggesting that grapefruit juice and/or its active compounds have ACAT have cholesterol-lowering and anti-atherosclerotic activity. HMG-CoA reductase also functions as a rate limiting enzyme in the cholesterol biosynthetic pathway [3, 68, 114]. The common HMG-CoA reductase inhibitors include statins such as simvastatin [3]. The effects of grapefruit juice in the expression of the enzyme HMG-CoA in diabetic rat models resemble those of HMG-CoA inhibitors. This is suggestive of statin-like effects of grapefruit juice in cholesterol metabolism.

4.7 Conclusion

This study was aimed at investigating the anti-diabetic and anti-dyslipidaemic effects of grapefruit juice in STZ-induced diabetic rat models. A type-1 diabetic rat model was successfully established, and the findings of the study indicate that indeed the administration of grapefruit juice does have anti-diabetic and anti-dyslipidaemic effects in STZ-induced diabetic rat models. The study suggests that grapefruit juice results in anti-diabetic effects by promoting glycogen synthesis, improving glucokinase activity, reducing G6Pase activity, as well as by reducing PEPCK enzyme activity. The anti-dyslipidaemic effects of grapefruit are suggested to occur by the elevation of HDL-C levels, improvement of the atherogenic index as well as through the down regulation of hepatic enzymes ACAT and HMG-CoA reductase. The management of diabetes mellitus and dyslipidaemia with GFJ may not only be associated with improving these diseases, but may also be associated with preventing and easing these diseases, and their complications.

4.8 Shortfalls of Study

While this study did indicate the potential beneficial effects of grapefruit juice administration in the treatment of diabetes mellitus and diabetic dyslipidaemia; it did not show the bioactive chemical constituents of the grapefruit that are responsible for the observed effects, as individual bioactive chemical constituents were not extracted and tested.

4.9 Future recommendations

- The effects of grapefruit should also be studied in type 2 diabetic rat models and effects compared to observed in type 1 diabetic rat models

- Methods that highlight the mechanism of action of grapefruit juice should be taken into consideration, so as to know how grapefruit juice brings about the observed effects.

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Appendix I

UNIVERSITY OF
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Animal Ethics Research Committee

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4 September 2013

Reference: 106/13/Animal

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

Dear Dr Owira

RENEWAL: Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for **2012/2013** on the following two-part project:

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

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

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

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

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Appendix II