Grapefruit Juice Ameliorates Nephropathy in Streptozotocin Induced Diabetes.

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Declaration

I **Julia Achieng' Hayangah** hereby declare that the dissertation entitled "***Grapefruit Juice Ameliorates Nephropathy in Streptozotocin Induced Diabetes*** " is the result of my own investigation, under the supervision of DR Peter M.O. Owira and that it has not been submitted in part or in full for any other degree or to any other university. Where use was made of the work of others, it is duly acknowledged and referenced in the text.

This study involved the use of animals and Ethical clearance was obtained from the university of KwaZulu-Natal ethics committee.

(Ethical clearance reference: **084/11/ Animal**)

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Supervisor:	signature	Date

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Dedication

I would like to dedicate this thesis to my grandmothers; Julia Onyango Odhiambo and Herina Oluoch Omollo as they were women of strength, character, foresight and compassion. May God rest there soul in peace.

Abbreviations

3-DG	3-deoxyglucosone
ACAT	Acyl-CoA: cholesterol acyltransferase
ACC	Acetyl CoA carboxylase
AGE	Advanced Glycation End Products
AMPK	AMP-activated Protein Kinase
ATP	Adenosine Triphosphate
BW	Body Weight
CDA1	Cell Division Autoantigen 1
CEL	N^{ε}-carboxyethyllysine
CML	N^{ε}–[carboxymethyl]-lysine
Creat	Creatinine
CTGF /CCN2	Connective Tissue Growth Factor
CTLA-4	Cytotoxic-T-Lymphocyte Antigen
D-45	Diabetic – 45mg STZ
D-60	Diabetic -60mg STZ
DAG	Diacylglycerol
DCT	Distal Convoluted Tubule
DKA	Diabetic Ketoacidosis
DN	Diabetic Nephropathy
DNA	Deoxyribonucleic Acid

ECF	Extracellular Fluid
ECM	Extracellular Matrix
Egr-1	Early Growth Response 1
EMU	Electron Microscope Unit
ESRD	End Stage Renal Disease
FAS	Fatty Acid Synthase
FBG	Fasting Blood Glucose
FFAs	Free Fatty Acids
FPI	Fasting Plasma Insulin
G6PD	Glucose-6-Phosphate Dehydrogenase
GADA	Glutamic Acid Decarboxylase
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GBM	Glomerular Basement Membrane
GD	Gestational Diabetes
GF	Grapefruit
GFJ- ND	Grapefruit Juice non-diabetic
GFJ	Grapefruit Juice
GFJ-D45	Grapefruit Juice -diabetic 45mg STZ
GFJ-D60	Grapefruit Juice diabetic 60mg STZ
GFR	Glomerular Filtration Rate
GIP	Glucose Dependent Insulinotropic Peptide

GK	Hexokinase IV /Glucokinase
GKRP	Glucokinase Regulatory Protein
GLP-1	Glucagon- Like Peptide 1
GLUT	Glucose Transporter
GP	Glycogen Phosphorylase
GPx-1	Glutathione Peroxidase-1
GS	Glycogen Synthase
GSK-3	Glycogen Synthase Kinase-3
GTT	Glucose Tolerance Test
H&E	Haemotoxylin and Eosin
HK	Hexokinase
HLA	Human Lymphocytic Antigen
HMG-CoA Reductase	3-Hydroxy-3-Methyl-Glutaryl- Coenzyme A Reductase
HNF	Hepatocyte Nuclear Factor
ICAM-1	Intracellular Adhesion Molecule -1
IDDM	Insulin Dependent Diabetes Mellitus
IFN-γ	Interferon Gamma
IL-2	Interleukin 2
INS-D60	Insulin- diabetic 60mg STZ
IPF-1	Insulin Promoter Factor 1
IR	Insulin resistance

ISSR	Inter-simple Sequence Repeat
LDLR	Low Density Lipoprotein Receptors
LF-L	Lactoferrin like polypeptide
MAPK	Mitogen Activated Protein Kinase Function
MCP-1	Monocyte Chemoattractant Protein-1
MDR1	Multidrug Resistant Protein 1
METC	Mitochondrial Electron Transport Chain
MGO	Methlyglyoxal
MIOX	Myo-inositol Oxygenase
MMP	Matrix Metalloproteinase
MODY	Maturity Onset Diabetes of the Young
MTS	Masson's Trichrome Stain
Na^+/ K^+ ATPase	Sodium Potassium Adenosine Triphosphate
NAD	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
ND	Non-diabetic
NDDM	Non-insulin Dependent Diabetes Mellitus
NeuroD1	Neurogenic Differentiation Factor 1
NF-κβ	Nuclear Factor- κβ
NO	Nitric oxide
OATP	Organic Anion Transporting Polypeptides (human)

oatp	Organic Anion Transporting Polypeptides (rat)
OCT	Organic Cation Transporter Protein (human)
PCT	Proximal Convoluted Tubule
PDGF	Platelet Derived Growth Factor
PEPCK	Phosphoenolpyruvate Carboxylase
P-gp	P-glycopoteins
PI_3-K	Phosphoinositide-3-kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PPH	Post Prandial hyperglycaemia
RAAS	Rennin-Angiotensin - Aldosterone- System
RAGE	Receptor for Advanced Glycated End Products
RAPD	Random Amplified Polymorphic DNA
ROS	Reactive Oxygen Species
SCAR	Sequence Characterized Amplified Region
SDH	Sorbital Dehydrogenase
SLGT	Sodium Glucose Co-Transporter
SREBP	Serol Regulatory Element Binding Protein
STZ	Streptozotocin
TNF-α	Tumor Necrosis Factor Alpha
TNF-β	Tumor Necrosis Factor Beta

UKZN	University of KwaZulu-Natal
VEGF	Vascular Endothelial Growth Factor
VIP	Vasoactive Intestinal Peptide
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization

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Abstract

Background

Diabetic Nephropathy (DN) is the leading cause of end stage renal disease and mortality in diabetic patients. Over the years, medicinal plants have been used to manage diabetes and its complications. Metformin, the synthetic analogue of galegine is used as first line therapy for Diabetes Mellitus (DM) but its use is contraindicated in patients with kidney dysfunction. The management of DN currently is limited to the use of anti-hypertensive agents; ACE inhibitors and angiotensin receptor blockers. Grapefruit juice (GFJ) has shown potential as an anti-diabetogenic agent because of its ability to ameliorate hyperglycaemia and dyslipidemia but its effect on fluid and electrolyte disturbance is not known. This study was designed to investigate the effect of GFJ on renal dysfunction in streptozotocin (STZ) induced male wistar rats.

Materials and Methods

Male wistar rats weighing between 250-300g were divided into 7 groups (n=7) and kept in cages for the treatment period of 8 weeks. Laboratory conditions of 12 hour light/dark cycle, temperature 25 ± 2^{0}C and humidity 50-55 % were maintained throughout the study period. Non-diabetic animals group 1 (Control) were treated orally with 1.0 ml /Kg BW of distilled water, while group 2 (ND-GFJ) were treated orally with 3.0 ml /kg BW of GFJ. The diabetic groups 3, 4, 5, 6 and 7 were starved overnight in preparation for the STZ injection. Fasting blood glucose concentration was obtained via tail prick before 45 mg or 60 mg of STZ was administered via a single injection in the peritoneal cavity. STZ was prepared by dissolving it in 0.2 ml of 0.1 M Citrate buffer at pH 4.5. Groups 3, 4 and 7 received 60.0 mg/ Kg BW of STZ while group 5 and 6 received 45 mg/kg BW of STZ. Three days following STZ induction, the diabetic state was confirmed by measuring fasting blood glucose and animals with glucose concentration greater than 6 mmol/L were included in the study. Group 4 (INS- D60) and group 5 (INS- D45) were additionally treated with 4.0 U/kg BW of insulin via subcutaneous injection (S.C) twice a day while Group 6 (GFJ-D45) and group 7 (GFJ-D60) were treated orally with 3.0ml/Kg BW of GFJ. Group 3 (D-60) were similarly treated with 1.0 ml /Kg BW of distilled water. Fasting blood glucose (FBG) and glucose tolerance tests (GTT) were done on days 1 and 58 respectively in all the treatment groups. Urine was collected for a period of 24 hours on day 59 and on the last day

animals were sacrificed by halothane overdose. Blood samples were obtained via cardiac puncture; kidney tissues were removed and preserved in formalin while the liver was snap frozen with liquid nitrogen and stored in a freezer (-80^{0}C) before analysis.

Results

Reduced plasma insulin was accompanied by decrease in body weight and an increase in FBG accompanied by polyuria, polydipsia and glucose intolerance in the non-treated diabetic animals compared to the control. Fasting blood glucose was significantly ($p<0.0001$) increased in the diabetic groups and treatment with GFJ or insulin lowered FBG in groups (GFJ-D45, GFJ-D60 & INS-D60) compared to the diabetic control (D60). GFJ significantly ($p=0.0034$) improved glucose intolerance in diabetic animals (GFJ-D60 & GFJ-D45) when compared to diabetic control groups D-60 & D-45 respectively. Hepatic glycogen content was reduced in diabetic animals ($P=0.024$) and treatment with GFJ significantly ($P=0.00016$) increased the glycogen concentration. In the non-diabetic group (GFJ-ND) treatment with GFJ significantly ($P=0.0013$) increased the glycogen concentration when compared to the control group. In the diabetic animals, decreased GFR was accompanied by Na^+ retention accompanied by low urinary K^+ and Cl^- concentration. Treatment with GFJ significantly ($p<0.05$) increased urinary Na^+ and K^+ and Cl^- in the diabetic group (GFJ-D60) but did not increase urinary Cl^- in non-diabetic group and consequently improved GFR in the diabetic group. Renal pathology showed structural changes in the glomerulus and treatment with GFJ had some reno-protective effect.

Conclusion

GFJ lowered the fasting blood glucose and improved glucose tolerance in the STZ-induced diabetic rats in a comparable manner to insulin treated diabetic rats. GFJ decreased Na^+ retention and increased GFR in the diabetic animals. This study suggests that GFJ could ameliorate nephropathy associated with diabetes mellitus. These results are the first to show that GFJ has renoprotective effects in STZ-Induced diabetic rats.

Key words: Grapefruit juice, diabetic nephropathy, hyperglycaemia

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The global flora consists of millions of different plant species that have served man well throughout history with over 20,000 species that are of huge importance because of their medicinal value [(1)]. Plants contain a number of bioactive compounds (phytochemicals) which have been exploited by man due to their desirable physiological and biochemical effects. In general, phytochemicals are not essential for sustaining life but they contribute significantly to optimal health [(2)]. The use of plants for medicinal purposes dates to antiquity, the first records were found in Egypt around 1550 BC and Babylon circa around 1770 BC in the code of hammurabi [(3) (4) (5)]. Ethno-botanical reports from around the world show that there are at least 800 plant species that may possess anti-diabetic properties [(6) (7) (8) (9) (10) (11)]. *Galega officinalis* is an example of a plant that was used in the medieval times to manage diabetes mellitus. Around the 1960s the bioactive compound galegine or isoamylene guanidine was extracted from the plant to produce phenformin and buformin but they were removed from the market in the 1970s because of their life threatening side effects [(12)]. Galegine was then modified to produce a synthetic analogue known as metformin, which has a lower incidence of lactic acidosis in comparison to its predecessors.

High costs associated with pharmacological agents used to manage diabetes and their associated side effects have seen people going back to the ways of their forefathers in the use of medicinal plants to manage common ailments. The safety and efficacy of these plants have not been established and this has lead researchers to look into ethno-medicinal claims and folkloric use of medicinal plants to ascertain their safety, efficacy and viability for the development into novel therapeutic. The use of complementary and alternative medicine has increased over the years because they are perceived to be safer, cheaper, and harmonious with the environment [(13)]. The search for novel anti-diabetics that would alter disease progression and or complications, be cost effective and have fewer side effects has captured the interests of researchers.

1.2 Background

Grapefruit is a classic example of a citrus fruit that is widely cultivated across the globe and its geographical distribution is in tropical and sub-tropical regions. This fruit is eaten as a whole or processed into thirst quenching juice that has gained global popularity because of its taste, nutritive value and potential health benefits associated with its consumption [(13) (14)]. Grapefruit's global production has increased steadily with approximately 6.23 million tonnes produced in 2010 [(15)]. It flourishes in tropical to subtropical regions in South Africa, United States [(16) (17)], Israel, Argentina, Cuba, and Mexico [(13)]. In South Africa, the geographical locations that favour citrus fruit production are: Eastern Cape, Kwazulu-Natal, Northern Cape, Mpumalanga and Limpopo with the latter 2 being the highest grapefruit producing areas [(17)] (Figure1). The percentage usage of the land allocated for grapefruit production in the 2 provinces is 37 % and 30 %, respectively (Table 1).

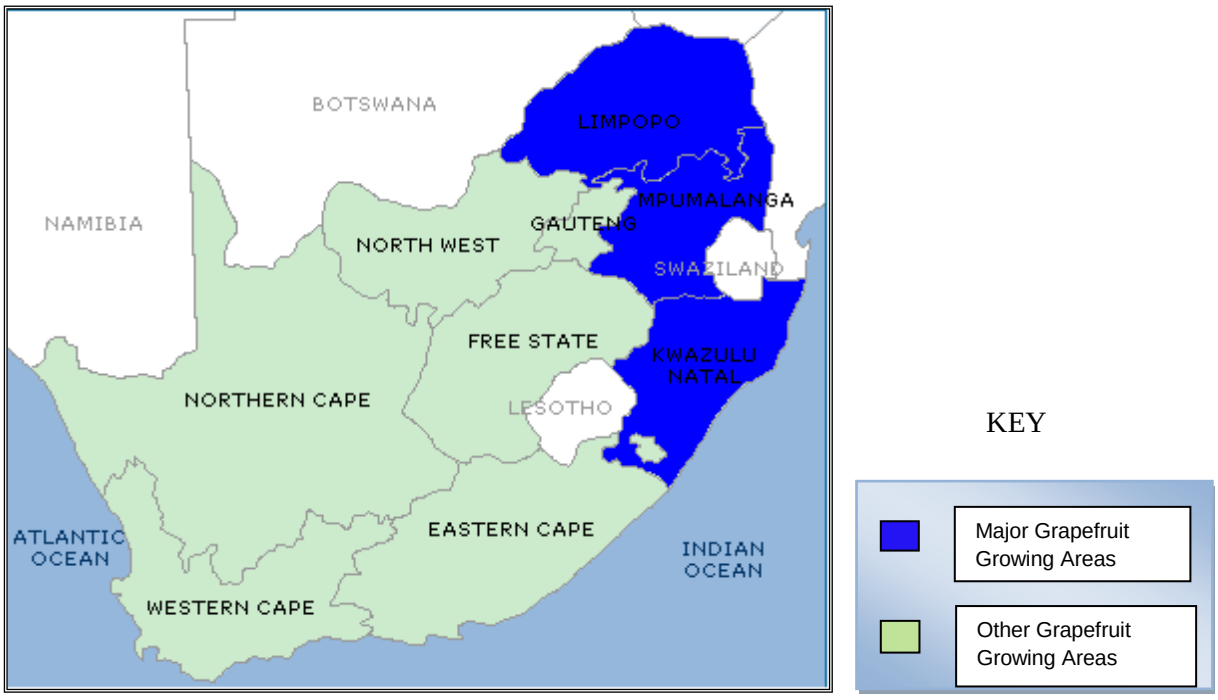


Figure 1: Citrus (Grapefruit) Growing Areas in South Africa [16]

Province	Area (HA)	Grapefruit Percentage
Limpopo	18,146	37%
Mpumalanga	12,050	30%
Kwazulu-Natal	3,824	20%
Eastern Cape	12,162	2%
Northern Cape	871	1%

Table 1: Percentage land usage for Grapefruit Production in South Africa [16]

1.3.0. Grapefruit

1.3.1. History of Grapefruit

The history of grapefruit dates back to the 16th century during the time of Christopher Columbus when he first brought the citrus biotypes to the Caribbean (18). Literature in botanical journals shows that the grapefruit's origin is in the tropical and sub-tropical regions in the West Indies. This citrus fruit was first mentioned, described and recorded by Patrick Browne in the in 17th century as the "forbidden fruit" or "smaller shaddock" (15) and was introduced to Florida in the 1820s (19). In 1830, James Macfayden a botanist, named a fruit from the botanical family rutaceae known as the "forbidden fruit" and gave it the latin name *Citrus paradisi* MACF. FAMILY Rutaceae (15) (20) (21).

Commercial production of grapefruit began in the late 1880 in the United States (22) and then it spread to other countries. According to chemotaxonomic evidence based on molecular biology and tests such as Random Amplified Polymorphic DNA (RAPD) and sequence characterized amplified region (SCAR) (23) shows that grapefruit has a total of 72 genetic markers (23) . Out of the 72 genetic markers found in grapefruit, forty-five of the markers can be traced back to the pummelo and twenty-seven to the sweet orange (24). This offered proof that the apomictical fruit originated from two different citrus fruits from the rutaceae family. Natural hybridization between the pummelo/ shaddock (*Citrus grandis*) which was the maternal progenitor and the sweet orange (*Citrus sinensis)* which was the pollen parent resulted in the grapefruit (GF) (20) (25) (26) (27) (28).

1.3.2. Grapefruit Morphology

The grapefruit tree is an evergreen tree that grows to a height of about 4.5 – 6.0 meters and its leaves are light green in colour when young and they become darker as they mature. The leaves on the grapefruit tree are characterised as simple and can be as long as 3-5 inches with large winged petioles and shiny with a broad base and a narrow apex. It is a flowering plant that produces white flowers and the fruits produced are large with thick rinds (hesperidium) that grow in clusters like gigantic grapes so it received the name grapefruit (Figure 2) [29] [30]. The fruits are pale yellow when ripe because of the breakdown of chlorophyll into xanthophyll and carotene which are yellow and orange respectively [30] with β-citraurin being the major xanthophyll in yellow grapefruits (Figure 2 and 3) [31].

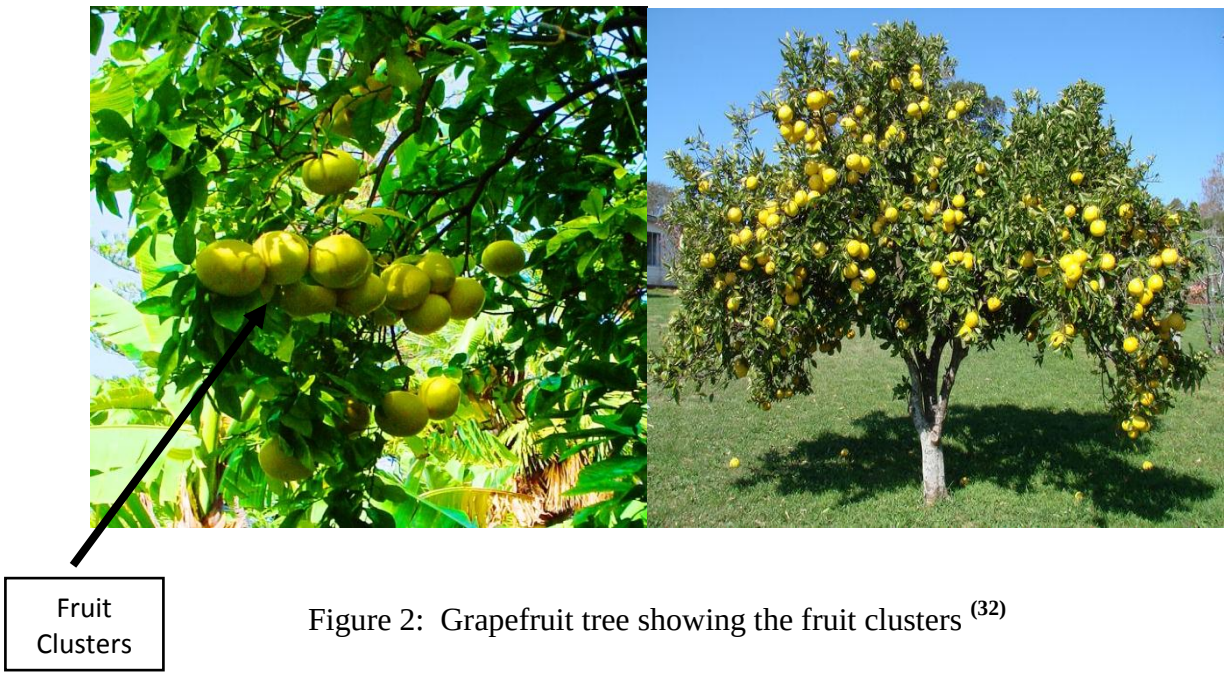


Figure 2: Grapefruit tree showing the fruit clusters **[32]**

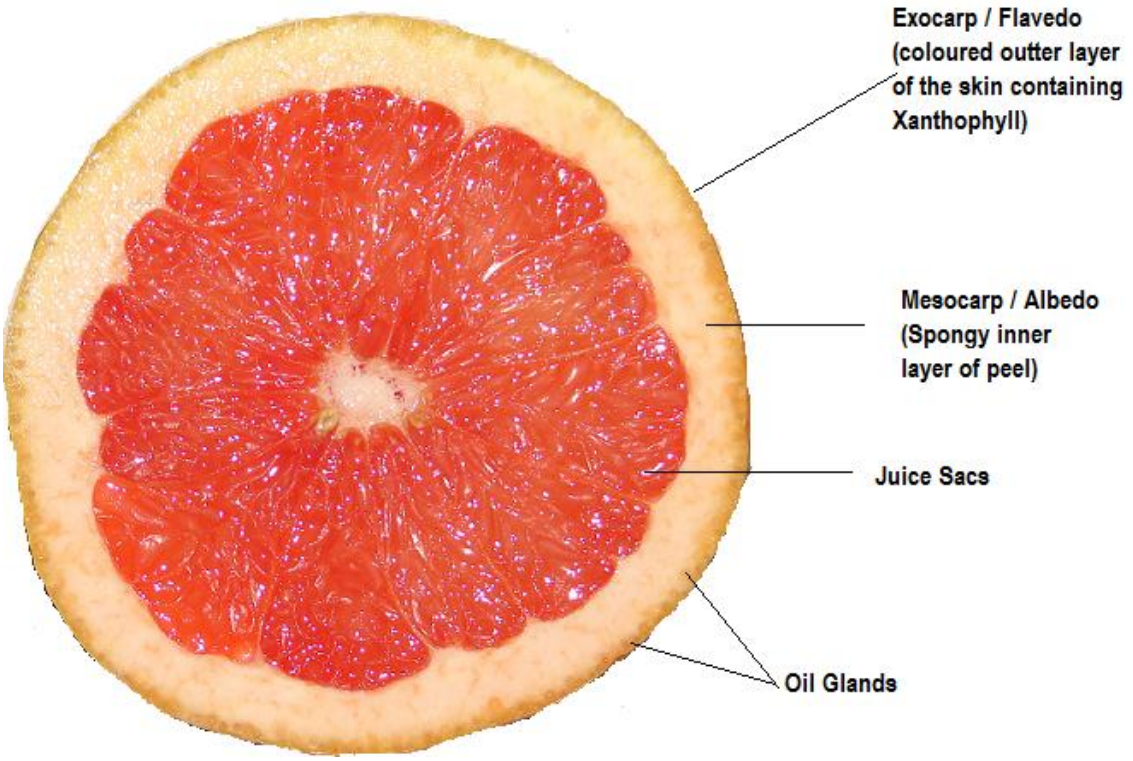


Figure 3 : Ruby Grapefruit Cross-section

1.3.3. Varieties of Grapefruit

All grapefruit cultivars are from the same ancestral tree and this has been confirmed by inter-simple sequence repeat (ISSR) markers [33]. Originally, the first grapefruit pulp was white but with the dawn of the 20th Century, citriculturists propagated different strains that varied from pink to slightly reddish in colour (Figure 4) [19]. The development of new grapefruit varieties was done through selected somatic mutations in the bud and or branches. The pink fleshed cultivars, arose from bud mutation [22] and mutation in the periclinal chimera branches of mash and waters gave rise to the Thompson cultivar (Figure 4) [34]. Varieties of grapefruit are as a result of artificial mutation by selection of desirable traits i.e. increasing redness, reduction in the number of seeds or improved taste [21] [23]. These mutations showed an increase in certain pigments like lycopene and carotene in the endocarp, rind and or pulp [34]. In South Africa, the cultivars commonly grown are star ruby and mash. Other varieties available in the global market can be categorised into two major groups according to the pulp colour and their seed content (Table 2).

GROUP	VARIETY	CHARACTERISTICS
Pale Yellow Pulp With Seeds ("White")	**Duncan** **MacCarty** **Triumph** **Imperial** **Walters**	**Oldest Grapefruit Cultivar**
Pale Yellow Pulp And Seedless ("White")	**Marsh**	
Pigmented Pulp (Generally Seedless)	**Thompson** **Flame** **Forester** **Red blush** **Ray ruby** **Star Ruby** **Henderson** **Ruby** **Davis**	**Pink** **Pink** **Pink** **Red** **Red** **Very red** **Very red** **Very red**

Table 2 : Grapefruit Cultivars [25] [34]

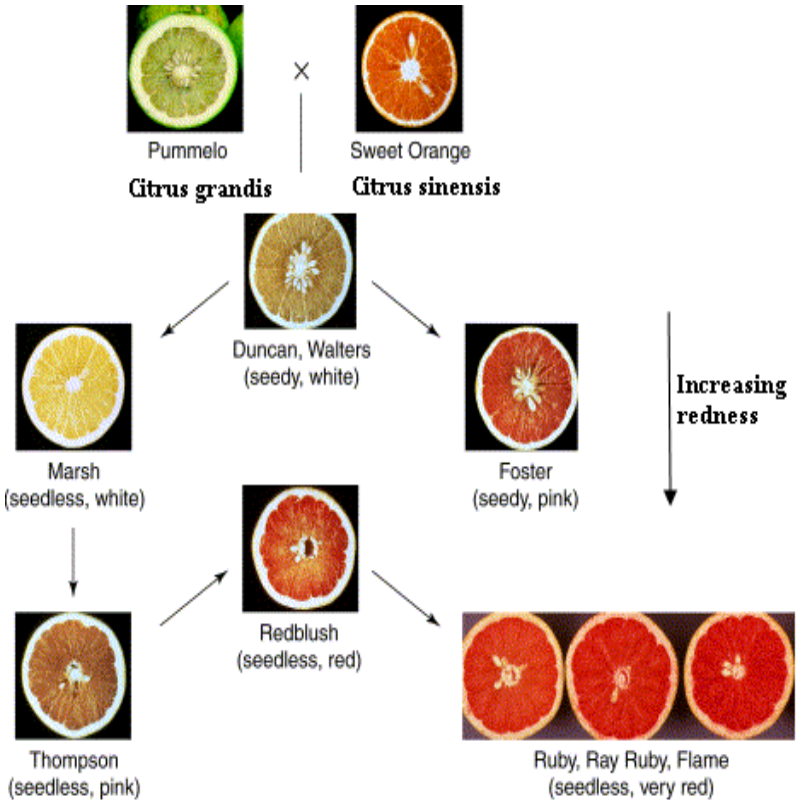


Figure 4: Grapefruit Cultivar Origin [23]

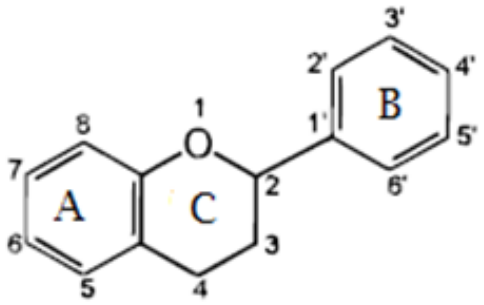
1.3.4. Medicinal Properties

The consumption of fruits and vegetables have gained importance due to their acclaimed effects that could help reduce the progression of certain diseases and or conditions that affect the endocrine system like diabetes and its related complications [35] [36]. Over the years grapefruit has received a lot of attention globally because of its acclaimed medicinal properties. GF has been used in the management of insomnia, anxiety, dysuria, cancer, anorexia, rheumatism and cardiovascular disorders [19]. Scientific research conducted on the whole fruit and its juice has suggested that this fruit could be having a plethora of beneficial medicinal effects which have justified and re-enforced its ethno-medicinal uses. Experimental evidence has shown that grapefruit possess significant hypoglycaemic [37], antioxidant [38], anticancer [31], anti-inflammatory, hypolipidemic [39], anxiolytic [40], antidepressant [40], antimicrobial [41], antifungal [41], cardio-protective protective effects [42] [43] and aids in weight loss [37] [44].

A study done by Fujioka *et al.* [44] showed that grapefruit could be beneficial to obese patients because it helps improve insulin resistance in patients with metabolic syndrome as compared to placebo [44]. Grapefruit juice (GFJ) has a high nutritive value, it provides 69% of the recommended daily allowance for vitamin C [19], which is a potent antioxidant, it is rich in folic acid, pectin, carotenoids and potassium [36] [45] . *In Vitro* studies have shown that the bioactive compounds in GFJ could elicit their pharmacological properties through the regulation of certain enzyme systems like the kinases [46] [47], phospholipases, ATpase, lipooxygenase and phosphodiesterases, quinine reductase and glutathione S-transferase [42] [48] .

1.4.0. Phytochemistry

Research conducted on grapefruit has led to the characterization and isolation of the different bioactive compounds found in the whole grapefruit (pulp, peel and juice). There are four different flavonoids found in the grapefruit namely flavonones, flavones, flavonols and anthocynanins [25]. Flavonoids are secondary plant metabolites which are diphenylpropane derivatives that are responsible for the taste, colour and a wide range of biochemical and pharmacological effects associated with grapefruit consumption [25] [35] [47] [49]. There are more than 5000 flavonoid compounds that can be categorized into 10 - 13 chemical groups [50] [51] with approximately 500 occurring in their aglycone form and the rest as O- or C-glycosides [52]. The basic flavonoid structure is a *benzo-γ-pyrone* (Figure 5) and addition of different functional groups like the hydroxyl, methoxyl, and *o*-glycoside to the basic structure [42] [51] leads to the formation of the different subclasses. Flavones, flavonols and flavonones are classified according to their state of oxygenation at carbon 3 [52].



Benzo- γ -Pyrone (Basic Flavonoid Structure)

Figure 5: Basic chemical structure of flavonoids **[42]**

Flavonoids are natural antioxidants and they have proven to be an efficient defence system in fighting against formation of free radicals [53] [54] such as hydroxyl (HO^-), nitric oxide (NO^-), peroxyl (ROO^-), superoxide anion (O_2^-), alkoxyl (RO^-) and singlet oxygen [52] [53] [55] [56]. Reactive oxygen species (ROS) have been associated with destruction of cell membranes, proteins, β-cells and deoxyribonucleic acid (DNA) [53]. The human body has enzymatic and non-enzymatic

defence systems that help prevent the damage of cells and tissues (Table 3) but their depletion or reduced production has been associated with degenerative conditions [53]. Flavonoids can prevent oxidative damage by chelating with transitional metals or by inhibiting the enzymes that are required for the initiation reactions [57] such as xanthine oxidase and protein kinase C (PKC) [53]. In addition, they can increase the body's intracellular glutathione, which is part of the endogenous oxidative defence system (up-regulation) or by protection of the existing mechanisms [47] [53].

Enzymatic Defence System	Non-Enzymatic Defence System
➢ Catalase metabolises hydrogen peroxide H_2O_2, ➢ Superoxide dismutase enzyme metabolises Superoxide anion (O_2^-) and lipid peroxidise inhibiting hydroxide formation	➢ Iron binding proteins transferin and ferritin (Iron is a Pro-oxidant metallic ion) ➢ Dihydrolipoic acid ➢ Reduced CoQ10

Table 3: Enzymatic and Non-enzymatic Defence System, **[52] [53] [57]**.

The flavonoids naringin and hesperidin are the most abundant bioactive compounds found in grapefruit (Table 4) [36] [58]. Naringin, hesperidin and limonin are responsible for the fruits bitter taste and the furocoumarins cause alteration of drug pharmacokinetics [25] [31] [48]. The quantity of phytochemicals found in grapefruit varies according to geographical location which determines the environmental conditions; the time of harvest determines the maturity and development, the variety, season and the methods used to process and treat the fruit after harvesting [34] [46] [48] [59] [60]. The highest concentration of flavonoids and furocoumarins are found during the early stages of development and this decreases with maturity of the fruit [36]. Amongst the different grapefruit

cultivars star ruby has the highest concentration of naringin, narirutin and neohesperidin (Table 4). The flavonoids in citrus fruits exist as flavones and flavanone glycosides [61] which are known to inhibit certain enzymes in varying degrees such as aromatase, xanthine oxidase, cyclooxygenase [62], lipooxygenase [62], cyclic GMP phosphodiesterase, cyclic AMP phosphodiesterase, angiotensin converting enzyme and thyroid peroxidase [52] [53] by as yet unknown mechanism. Naringin and hesperidin are glycosides that are hydrolyzed at the intestinal brush border to their various aglycones naringenin, hesperetin and a sugar [52] by the intestinal enzymes naringinase and α-L-rhamnosidase respectively [58] [63] [64](Figure 6 and 7). Glycosides differ from the aglycones in that they lack a sugar moiety attached to the hydroxyl group at C-3 or C-7 [65]. The common sugar moieties on the glycosides are D-glucose and L-rhamnose [65] and they make the aglycones more soluble [50]. Naringenin and hesperetin appear to be the pharmacologically active forms but their actions differ significantly in terms of their pharmacologic activities that could be of clinical significance (Table 6).

Composition		Grapefruit Cultivars			
		Star Ruby	Rio Red	Ruby Red	Handerson
Organic Acids	Example	g L^{-1}±SD	g L^{-1}±SD	g L^{-1} ±SD	g $^{L-1}$ ±SD
	Malic acid	1.84±0.02	2.97±0.56	2.11±0.01	2.57±0.48
	Citric acid	23.89±0.69	18.75±0.53	21.74±1.07	22.46±0.65
	Ascorbic acid	0.43±0.08	0.31±0.04	0.33±0.02	0.41±0.01
Sugar	Sucrose (Main sugar)	34.99±0.33	32.58±0.56	24.45±0.25	33.44±0.56
	Fructose	22.32±0.29	25.64±0.33	23.29±0.15	26.50±0.20
	Glucose	23.30±048	23.68±0.05	22.12±0.08	26.56±0.24
Phenolic Composition		mg L^{-1}±SD	mg L^{-1}±SD	mg L^{-1}±SD	mg L^{-1}±SD
Flavonoids	Narirutin	120.06±0.99	96.12±1.91	63.80±0.70	102.75 ±0.12
	Naringin	464.13±1.37	320.41±1.70	270.21±1.86	352.67±0.57
	Hesperidin	8.94±0.05	10.25±0.15	9.21±0.25	8.47±0.27
	Neohesperidin	24.24±0.26	19.11±0.25	15.96±0.52	14.72±0.72
Hydroxycinnamic	P-coumaric acid	15.84±0.22	13.70±0.04	13.90±0.012	16.30±0.14

Table 4: Potential Bioactive compounds found in Grapefruit Juice [35] [46] [66] [67]

In addition to flavonoids grapefruit juice also possesses inorganic elements that play an essential part in the maintenance of biological processes in the body because they form part of

metalloproteinases and some can preclude development of certain complications associated with diabetes [68]. Several studies have highlighted the importance of zinc and selenium in ameliorating complications associated with diabetes due to their insulin mimetic properties [69] [70] [71].

Inorganic Elements	Copper
	Calcium
	Iron
	Magnesium
	Manganese
	Phosphorus
	Potassium
	Selenium
	Zinc

Table5: Inorganic elements present in grapefruit juice [72]

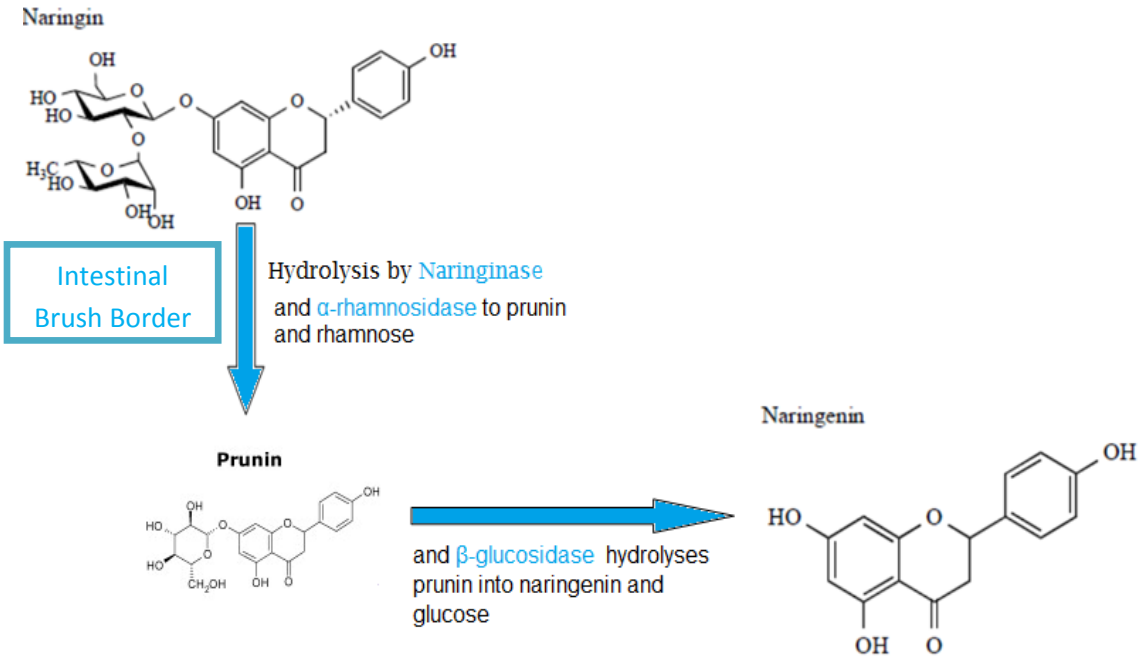


Figure 6: Enzymatic hydrolysis of naringin to its active aglycones, naringenin [73] [58] [63] [74].

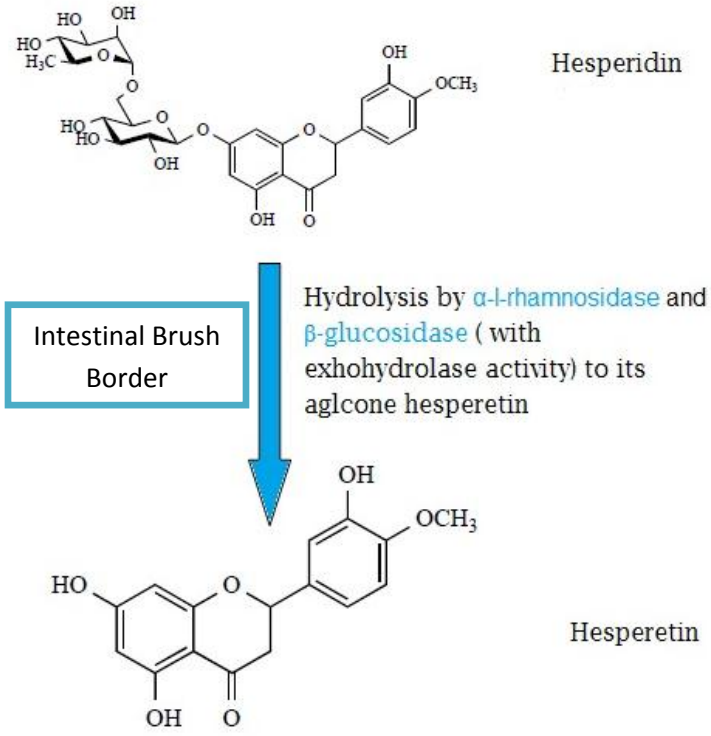


Figure 7: Enzymatic hydrolysis of hesperidin to its active aglycones, hesperetin **(73) (58) (63) (74)**.

1.4.1 Flavonoid Glycosides Metabolism

Flavonoids are metabolised in the liver, intestinal wall and lumen before their metabolites are absorbed into the systemic circulation (65). Gastrointestinal bacteria like *Bacteroides* JY-6 (75), *Streptococcus faecium* VHG-1, Streptococcus sp. FRP-17, *Escherichi coli* HGH21 and HGH6 are known to produce enzymes that hydrolyse glycosides to their various aglycones (Figure 6 and 7). The enzymes lactase phlorizinhydrolase (75) and α-rhamnosidase (65) are associated with glycoside deglycosylation. Some of the absorbed flavonoids undergo conjugation reactions by hepatic enzymes but a majority of the flavonoids are broken down by the intestinal micro flora (53). The aglycones are degraded further through c ring fission (52) (76) and the products are absorbed into the blood stream and they undergo methylation, glucuronidation, sulfation (75) and excreted in the faeces and or urine as conjugates (77) (78).

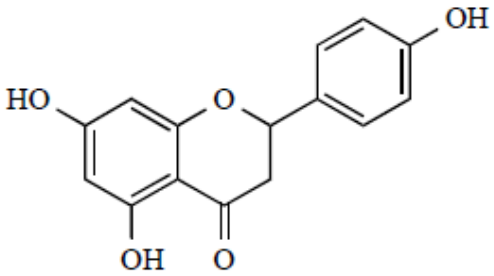
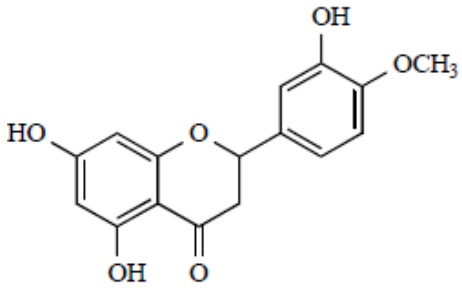
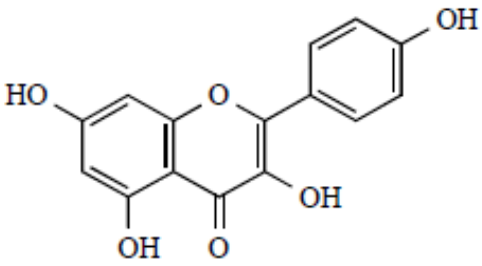
Flavonoid subclass	Structure	Examples	Known Properties / references
Flavanone		**Naringenin** 4',5,7-trihydroxy flavanone	• ↓Activity of hepatic glucose-6-Phosphatase and Phosphoenolpyruvatecarboxykinase [79] • Inhibition of renal and intestinal Na^+ glucose Co-transporters [80] .
		**Hesperetin** 7-rhammnoglucoside	• Hesperidin causes Upregulation of hepatic glucokinase, PPAR γ, and adipocyte GLUT-4 [50]. ↓activity of glucose-6-phosphate dehydrogenase and phosphatidatephosphohydrolase [50]. • Hesperitin: ↓HMG-CoA reductase and ACAT.
Flavonol		**Kaempferol** 3,4',5,7-tetrahydroxy flavones	• ↓ Blood glucose and stimulates glucose uptake by altering intrinsic activity of glucose transporter[[79]

Table 6: Flavonoids Found In Grapefruit with potential anti-diabetic properties **[50] [61]**

1.5.0. Grapefruit Drug Interactions

The drug interactions associated with GFJ consumption were first discovered accidentally in 1989 during a study that was examining the interaction of ethanol and felodipine when grapefruit was used to mask the taste of ethanol [21]. It was observed that GFJ altered the pharmacokinetics of felodipine and subsequent studies have confirmed that this effect is indeed a cause of concern because many patients take their medication with GFJ and this can lead to life threatening toxicity due to increased drug bioavailability [25] [36] [48]. The interactions between GFJ and prescription drug is limited only to oral dosages and no interactions have been noted when the drugs are administered intravenously [21]. GFJ has been known to interact with up to 50% of prescription drugs such as; calcium channel blockers, cholesterol lowering drugs ; statins, protease inhibitors, benzodiazepines and phosphodiesterase inhibitors [21] [36] [81] [82] [83] (Table 7).

1.5.1. Mechanism of Grapefruit Juice Interactions

The major drug metabolising enzyme systems in the body are the Cytochrome P450 (CYP450) predominantly found in the liver and small intestines with drug specific isoforms [48]. The CYP450 enzyme systems are responsible for the biotransformation of a number of drugs before they reach the systemic circulation by first pass hepatic metabolism. GFJ has shown significant activity in altering the functioning of xenobiotic metabolizing enzymes (CYP450) and or transporter proteins in the intestines [84] [85]. Bioactive compounds found in GFJ such as furanocoumarins are potent inhibitors of CYP450 system [86] especially the CYP3A4 isoform which is abundant in enterocytes and is responsible for metabolizing > 50% of prescription medication [19] [87]. GFJ inhibits the cytochrome CYP3A4 and alters the functioning of drug transporter proteins namely P-glycopoteins (P-gp), human organic cation transporter protein (OCT) and human organic anion transporting polypeptides (OATP) [48] [83] [84]. Bergamottin and 6'7'-dihydroxybergamottin are example of furanocoumarins (Figure 8) that have been identified and isolated from GFJ and have been found to irreversibly inhibit CYP3A4 enzyme by mechanism based inactivation [88] causing an increase in the plasma concentration of CYP3A4 substrate drugs (Table 7) [21] [31] [89] [83] [88] [89]. P-glycoprotein is an efflux pump located on the apical brush

border of enterocytes that transports drug substrates from the enterocyte into the gut lumen thus decreasing oral bioavailability [(90)]. The effect of grapefruit juice on the efflux pump is very controversial with reports ranging from inhibition to its activation [(91)]. The study done by Wang *et al.* [(85)] showed that bergamottin inhibited the P-gp efflux pump in a concentration dependent manner resulting in increased oral bioavailability of digoxin.

OATP is a drug uptake transporter that is from the solute carrier super family (SLC). Its expression is similar to that of P-gp and it plays a major role in the transportation of drug substrates into the cell. A study done by Dresser *et al*. [(91)] showed that the furanocoumarin (6'7'-dihydroxybergamottin) inhibited the activity of rat organic anionic transporting polypeptide 3 and 1(oatp3 and oatp1) and human OATP activity resulting in increased plasma concentration. In addition GFJ flavonoids naringin and hesperidin have also been implicated in the inhibition of OATP1A2 *in vitro* [(92)]. GFJ furanocoumarins monomers, dimer [(93)] and naringenin can therefore alter the activities of p-glycoprotein / Multidrug resistant protein 1(MDR1) and the OATP which play a part in altering drug pharmacokinetics [(82) (94)].

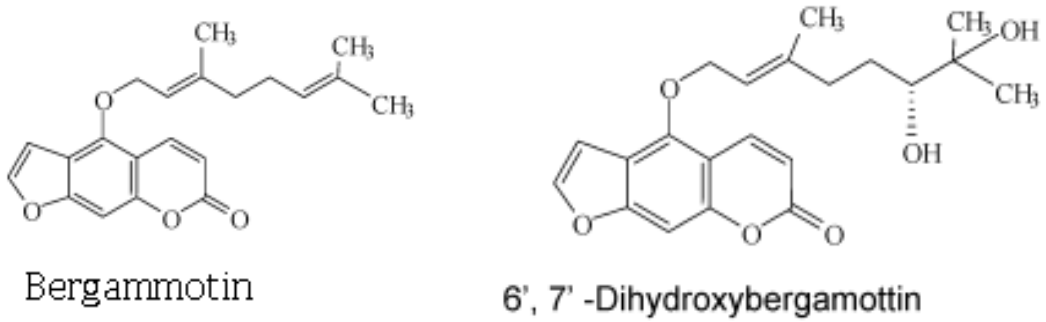


Figure 8: Chemical structure of Bergamottin and 6'7'-dihydroxybergamottin **[(36)]**

Class of Drug	Example of Drug	References
Antiarrhythmics	Amiodarone, Propafenone, Dronedarone	(21) (25)
Sedative Hypnotics/ Anxiolytics	Midazolam, Triazolam, Buspirone	(21) (81) (88) (93) (77)
Antiepileptics	Carbamezepine	(21) (31)
Antihistamines	Fexofenadine	(21) (25)
Antimalarials	Artemeter, Primaquine	(21)
Antipsychotics	Pimozide	(21)
Calcium channel blockers	Felodipine ,Verapamil, Amlodipine, Nicardipine, Nimodipine, Nifedipine	(21) (25) (31) (81) (88) (93)
Cytotoxic Agents	Nilotinib, Lapatinib, Dasatinib	(21)
HMG-CoA inhibitors	Artovastatin, Simvastatin, Lorvastatin	(21) (25) (31)
Hormones	Ethinylestradiol	(86) (93)
Immunosuppressant	Ciclosporin, Tacrolimus, Sirolimus	(21) (81) (88) (86) (77)
Opioids	Oxycodone	(21) (83)
Phosphodiesterase- 5-inhibitor	Sildenafil, Tadalafil, Vardenafil	(21) (86)
Protease inhibitors	Saquinavir, ritonavir	(21) (86) (88) (93)
Biguanide	Metformin	(37)

Table 7: List of drugs that grapefruit increases their serum concentration.

1.5.2. Flavonoids with Potential anti-diabetic Activity.

Flavonoids like naringinin and hesperitin found in GFJ have shown to have an array of pharmacological activities that would be beneficial to a diabetic patient. Naringin has been shown to reduce the activities of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6- phosphatase key enzymes in the gluconeogenesis process in the liver [(79)] and it has the potential to ameliorate diabetic artherogenic dyslipidemia [(39)]. The ability of hesperitin and naringenin in inhibiting key enzymes involved in the synthesis of cholesterol such as 3-hydroxy-3-methyl-glutaryl- Coenzyme A reductase (HMG-CoA reductase) and acyl-CoA: cholesterol acyltransferase (ACAT) [(95)] [(96)] suggests that they could be potent hypocholesterolemic agents. Chanet *et al* [(97)] showed that consumption of naringin significantly reduces diet-induced atherosclerosis in mice [(88)]. This is because naringenin increases the expression of low density lipoprotein receptors (LDLR) in HepG2 cells by activation of mitogen–activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI_3-K) mediated uptake of serol regulatory element binding protein (SREBP) resulting in reduced low density lipoprotein accumulation [(97), (98)].

Experimental evidence suggests that naringenin can also inhibit the microsomal triglyceride transfer protein activity causing a decrease in blood lipids and fatty liver [(95)] [(97)]. In addition to that, the aglycone hesperitin improved the atherogenic index in experimental rats [(99)]. The hypoglycaemic effects of GFJ bioactive constituents have been reported, suggesting that naringenin can decrease glucose uptake in the renal and intestinal tissues because it inhibits the sodium – glucose co-transporter 2 (SGLT 2) [(80)]. In the skeletal muscle, naringenin increases glucose uptake via activation of AMP-activated protein kinase (AMPK) [(100)] significantly reducing the plasma glucose concentration. Other mechanisms by which GFJ lowers blood glucose concentration and ameliorates complications associated with diabetes mellitus are still under investigation.

1.6.0. Diabetes Mellitus

1.6.1 Epidemiology

The public health sector in both the developed and developing countries alike are plagued with the burden of non-communicable diseases like diabetes. Studies show that diabetes will affect many people in developing countries because of lack of effective and affordable treatment [101]. Global data shows that approximately 130 million [102] people are suffering from diabetes mellitus and by the year 2030 the number of people suffering from diabetes will be in the region of 366 million [103] and this could rise to 522 million as stated during the world diabetes day in Brussels on the 14th of November 2011 [104]. Epidemiological data shows that diabetes and its related complications will have negative impact on the general population due to the unprecedented increase in healthcare costs associated with their management which can significantly cripple the struggling economies and if not managed properly leading to increased mortality rates in affected populations.

In South Africa, it is estimated that 11.5 million people suffer from diabetes mellitus [103] [105] and this figure is expected to rise due to prevalence of obesity, sedentary lifestyles, urbanization [106] [107] and physical inactivity [103] . The population groups in South Africa mostly affected are the black, coloured and Indian communities. Shaw *et al* [107] predicts that South Africa's national prevalence by the year 2030 will be at 4.9% with a mean yearly increment of 18,000 people affected with diabetes [107].

1.6.2. Definition and Classification

According to the 1999 World Health organisation (WHO) consultation report, diabetes mellitus was defined as "a metabolic disorder of multiple etiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both" [108] . Diabetes mellitus can be categorised into two major groups depending on the exogenous insulin requirements. The two major groups are type 1 which is also known as insulin dependent diabetes mellitus (IDDM) and type 2 which is the non-insulin dependent diabetes mellitus (NIDDM) [102] [109]. Type 2 diabetes is the most common form and it accounts for 90-95% of all diabetic cases affecting the general

population while Type 1 accounts for 5-10% and generally affects children [101] [102] [110] [111] . Other forms of diabetesidentified are gestational diabetes (GD) and Maturity onset diabetes of the young (MODY). GD develops during pregnancy causing dysfunctions in the carbohydrate metabolism due to acquired insulin resistance resulting in chronic hyperglycaemia in the mother [112]. It is characterised by glucose intolerance and it is a risk factor for the development of type 2 diabetes in pregnant women postpartum [112].

Maturity onset diabetes of the young (MODY) on the other hand occurs in non-obese adolescents. MODY also referred to as monogenic diabetes and can be classified according to where the single point mutation occurred, i.e. if it occurred on the metabolic enzyme or the transcription factors. Mutation of the glucokinase enzyme leads to MODY subtype 2 (MODY 2). The other subtypes of MODY occur due to mutation of different transcription factors namely; hepatocyte nuclear factor (HNF) - 4alpha, HNF-1alpha, HNF-1 beta, insulin promoter factor (IPF-1), neurogenic differentiation factor 1(NeuroD1) accounting for the other 5 subtypes [113] [114] [115]. MODY2 is associated with reduced glucose induced insulin secretion that is accompanied by a reduction in glycogen storage in the liver resulting in hyperglycaemia [116].

1.7.0 Aetiology and Pathophysiology of Diabetes Mellitus

Diabetes affects the endocrine tissues of the pancreas leading to a disruption in the negative feedback mechanism of the body that is involved in blood glucose regulation. Destruction of the pancreatic β-cells can lead to an absolute or relative insulin deficiency as seen in diabetic patients. A number of factors have been known to cause the destruction and or dysfunction of pancreatic β-cells located in the islets of langerhans. The identified factors include; autoimmune diseases, genetic defects of the pancreatic β-cells, defects in insulin action, environmental toxins, viruses and drugs or chemical agents like streptozotocin and alloxan [109]. Streptozotocin (STZ) is a nitrosourea analogue that has high specificity for the pancreatic β-cells. It was first isolated from *Streptomyces achromogenes* a soil microbe and used for the treatment of pancreatic β-cell carcinoma [117] . It works by destroying the β-cells through the

generation of super oxide anions that act on the mitochondria causing an increase in the activity of the enzyme xanthine oxidase [118] causing total or partial destruction of the pancreatic β-cells. The administration of streptozotocin 60mg/BW and 45mg/BW has been shown to create type 1 and type 2 diabetic models respectively. The difference in the dosage causes i.e 60mg/BW causes complete destruction while 45mg/Kg causes partial destruction of the pancreatic β-cells. The destruction leads to a significant drop in insulin levels and ultimately no insulin production and this explains their use in the development of type 1 and 2 diabetic models in the experimental animals [118].

The clinical symptoms associated with diabetes are because of chronic hyperglycaemia and the response of the body in trying to correct the imbalance. The normal range for blood glucose concentration is around 3.3 mmol/L - 8.3 mmol/L depending on the fed or fasted state of the person [119]. Diabetic patients experience impaired Fasting Blood Glucose (FBG) and glucose intolerance with FBG greater than 6.7 mmol/L [120]. The glomerulus filters a large volume of glucose from the blood stream and it is usually reabsorbed in a multiple step process via sodium glucose co-transporter 2 (SGLT2) in the proximal convoluted tubule and GLUT-2 transporters at the basolateral membrane and back into the blood stream [121]. Glomerular filtration of glucose and subsequent reabsorption increases in a linear manner with an increase in concentration until a threshold is reached at approximately 10 mmol/L [121] [122].

The renal tissues of diabetic patients cannot maintain the FBG at approximately 5.6 mmol/L due to increased glucose reabsorption [122]. When glucose concentration is above the theoretical threshold of 11 mmol/L, saturation of the glucose reabsorption transport system occurs causing excess glucose to be excreted in the urine (glycosuria) Figure 9 [122] [123] .Glucose excretion in the urine is accompanied by increased loss of water and electrolytes (polyuria) as observed in patients suffering from diabetes. This process causes dehydration and the body attempts to correct the imbalance by increasing thirst (polydipsia) [124].

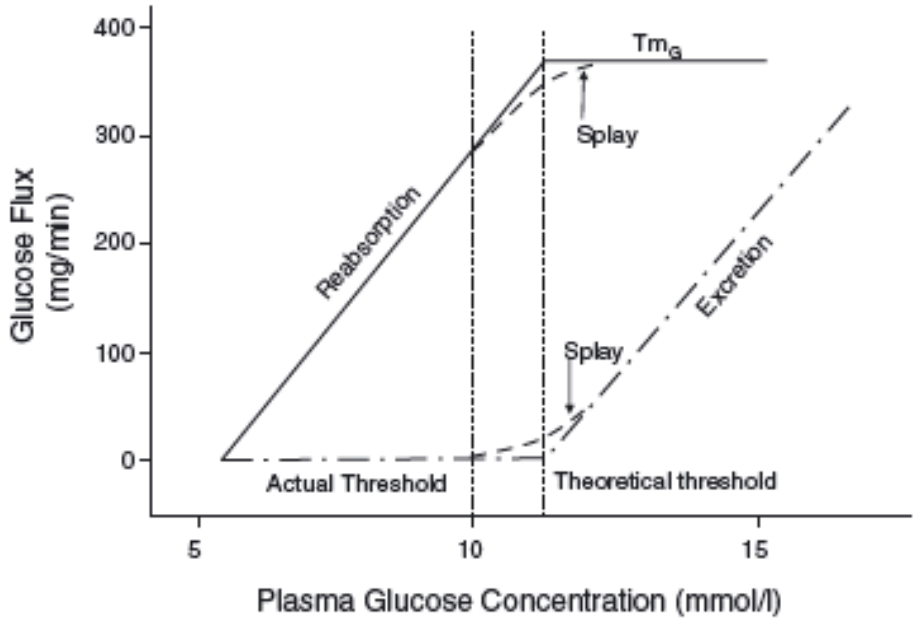


Figure 9: Renal glucose handling adapted with permission from Defronzo *et al* 2012 **(122)**.

1.7.2. Type 1 Diabetes

In type 1 diabetes, multiple factors can cause destruction of the β-cell as previously discussed but autoimmune response in genetically predisposed children is the most common form that affects type 1 diabetic patients resulting in an absolute deficiency of insulin production [(111) (125) (126) (127)]. Genetically predisposed individuals possess the human leukocyte antigen genotypes (HLA) on the DR and DQ especially on HLA DR3 and HLA DR4 [(111) (124) (128)] making them more susceptible to the development of type 1 diabetes after an environmental trigger. HLA-chromosome 6 is the genetic portion that is linked with inheritance from parent to the child [(111) (125)]. Genetic susceptibility involves HLA and other genetic factors like the insulin gene on chromosome 11 and cytotoxic-T-lymphocyte antigen (CTLA-4) on chromosome 2 [(125)]. Some of the environmental triggers identified are enteroviruses, cox sackie, congenital rubella, toxins from foods and or chemical agents. They can trigger an autoimmune response in a genetically susceptible individual who has one or more antibodies to the beta antigens that will eventually lead to an inflammatory process (insulitis) [(111)].

Insulin, glutamic acid decarboxylase (GADA) and protein tyrosine phosphatise (IA2) are autoantigens, that have been implicated in the pathogenesis of type 1 diabetes [111] [129]. Studies have shown that T-cells, macrophages / dendritic cells, β-cell autoantigens and cytokines are implicated in the pathological development of type 1 diabetes [129] [130]. The failure of the immunoregulatory system causes auto-reactive T-cells to activate Th1 that promotes inflammatory immune responses and suppresses Th2, which is a subset of the $CD4^+$ T–cells responsible for humoral immunity [131]. The T- cell lymphocyte Th1 causes the secretion of inflammatory mediators namely; interleukin 2 (IL-2), interferon gamma (IFN-γ), tumor necrosis factor beta (TNF-β), tumor necrosis factor alpha (TNF-α) [132] [131]. These cytokines cause the recruitment of circulating leukocytes into the islet cells and this leads to the activation of macrophage elimination of antigen bearing cells. This is because the autoantibodies attack the β- cell specific autoantigens on the cell surface leading to the activation of cytotoxic T cells by IFN-γ and IL-2 resulting in an inflammatory response that causes pancreatic β-cells destruction [111] [127].

1.7.3. Type 2 Diabetes

In type 2 diabetes, genetic predisposition and a combination of environmental factors intertwined together lead to the dysfunction and eventual destruction of the pancreatic β-cells [133]. Some of the genetic variants that are associated with type 2 diabetes are *AMP1/GBP28* gene that encodes for adiponectin [133] in the Japanese population, *KNCNQ1* gene that encodes for pore forming subunit in voltage gated K^+ channels in East Asians and Europeans [134]. These genetic variations in addition to environmental factors like diet, obesity and sedentary lifestyle increase the risk for development of type 2 diabetes. Type 2 diabetes is characterised by hyperglycaemia, declining beta cell function [135], impaired glucose tolerance, insulin insufficiency [136] and reduced insulin mediated glucose uptake and utilization (insulin resistance) [102]. Insulin resistance (IR) is a major problem because of the fatal clinical consequences associated with its development. Resistance to the action of insulin in the hepatic tissues causes increased glucose and VLDL production, in the adipose tissues, increased release of free fatty acids (FFAs) and in the skeletal muscle reduced glucose utilization [119]. A number of abnormalities on the insulin receptors found in different

tissues, increased FFAs accumulation, obesity, diseases like Cushing's syndrome, acromegaly, glucogonoma and or genetic defects can cause insulin resistance. In diabetic patients , IR can develop due to accumulation of intracellular fatty acid metabolites that prevent the activation of phosphoinositide -3- kinase (PI_3-kinase) resulting in impaired glucose uptake and other effects of insulin receptor signalling [137] [138]. This can cause severe problems that could lead to the development of hyperglycaemia induced tissue damage, dyslipidemia, CVD, hypertension, metabolic syndrome and or progression of β-cell dysfunction.

1.7.3.1. Pancreatic Beta (β) Cell Dysfunction

The pancreas is an organ with exocrine and endocrine tissues that plays a major role in the production of hormones that are responsible for blood glucose regulation [139]. This organ consists of two major segments namely the acini and islets of Langerhans. The islets of Langerhans comprise of four distinct cells namely the alpha, beta, delta and F-cell. The β-cells form bulk of the cells followed by the alpha and delta cells respectively [140]. The islets of Langerhans' major role is the production of insulin, glucagon and somastatin released from the beta, alpha and delta cells respectively. The β-cell is susceptible to destruction by chemical agents, hyperglycaemia and lipotoxicity and or disease [141].

In type 2 diabetes, pancreatic β-cell toxicity results from chronic hyperglycaemia and increased intra–islet lipid accumulation [135]. The generation of reactive oxygen species (ROS) due to glucose toxicity, a dysfunctional mitochondrial system and formation of advanced glycation end products (AGE) causes increased oxidative stress that lead to the progression of β-cell dysfunction [141]. This is because the pancreas lacks a natural defence system against oxidative stress due to lack of the glutathione peroxidase-1 (GPx-1) gene [141] [142] [143]. The loss of β-cell mass leads to insufficient insulin production and this causes decreased glucose transport in fat and, muscle cells accompanied by increased fatty acid accumulation [138] [144] .

1.7.4. Glucose Homeostasis

1.7.4.1. Insulin

Insulin is an anabolic, pleiotropic hormone that works by controlling the expression of different genes encoding for metabolic enzymes required for glycolysis and inhibition of glycogenolysis, and the gluconeogenic processes [119] [138]. A rise in blood glucose concentration above 8.3 mmol/L and an increase in amino acid levels after a meal stimulate the release of insulin from the pancreatic beta cells through an intricate process [145]. This multiple step process is triggered by movement of glucose via facilitative diffusion aided by GLUT-2 transporter proteins located on the surface membrane of the pancreatic β-cells [138]. Once in the cell, glucose is phosphorylated to glucose -6- phosphate catalysed by the glucokinase enzyme but this process is blocked in people with genetic mutation of the glucokinase gene and this results in reduced glucose stimulated insulin secretion [146] .The metabolism of glucose (glycolysis) causes the generation of ATP and a rise in the ATP/ADP ratio causing the inhibition of the ATP sensitive K^+ channel. Intracellular build-up of K^+ causes membrane depolarisation and the opening of voltage sensitive Ca^{2+} channels [147] [148]. The influx of calcium into the cells causes insulin containing granules to fuse with the plasma membrane resulting in the release of insulin into the circulation (Figure10) [116] [147].

Hormones released after a meal cause the second phase of insulin release. These hormones are glucagon like peptide -1 (GLP-1) and vasoactive intestinal peptide (VIP) [148]. The activation of phospholipases and protein kinase C by acetylcholine causes the stimulation of adenylyl cyclase activity that leads to the activation of protein kinase A which potentiates insulin secretion [149]. This process of insulin secretion does not occur in the fasting state or in response to hypoglycaemia.

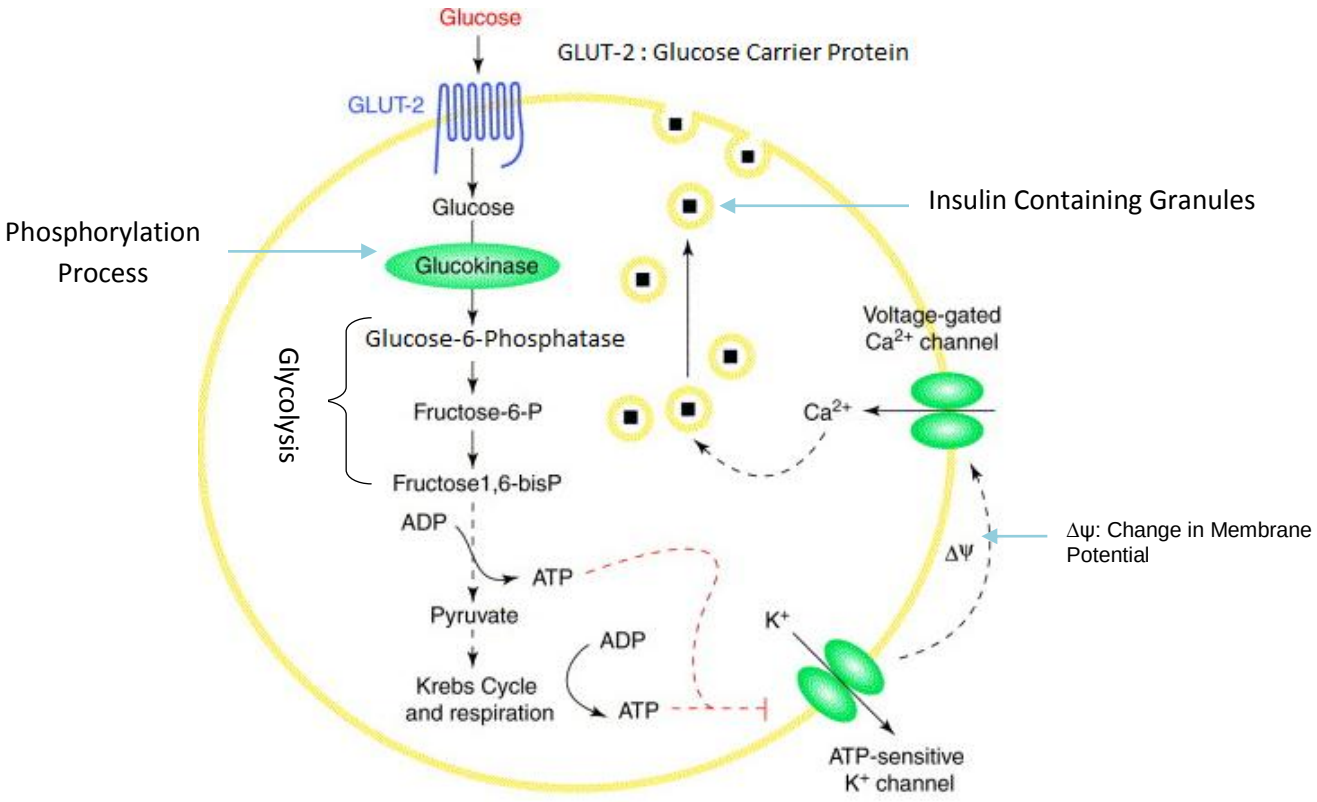


Figure 10: Mechanism of insulin secretion in the pancreatic β-cells modified and adapted from Rolland *et al* 2001 [(150)]

1.7.4.2. Anabolic Effects of Insulin

Insulin actions are initiated by its binding to the insulin receptors that are located on the plasma membrane in the liver, skeletal muscle and adipose tissues. The insulin receptors belong to a family of receptor tyrosine kinases that include insulin like growth factor and insulin- receptor- related - receptor [(138)]. The insulin receptor is a heterotetrameric glycoprotein consisting of two α and β subunits [(151)]. The binding of insulin on the α-subunit leads to auto phosphorylation of the tyrosine residue on the β subunits activating the intracellular signalling pathways responsible for insulin metabolic and mitogenic actions via activation of PI_3-Kinase pathways [(138) (145) (151) (152)]. PI_3-Kinase catalyses the phosphorylation of phosphoinositides to phosphatidylinositol-3-phosphates resulting in the AKt / PKB activation and consequent gene expression and transcription of glucokinase, pyruvate kinase , fatty acid synthase and acetyl CoA carboxylase and the inhibition of transcription encoding for glucose -6- phosphatise and fructose 1-6- biphosphate (Figure 11) [(138)].

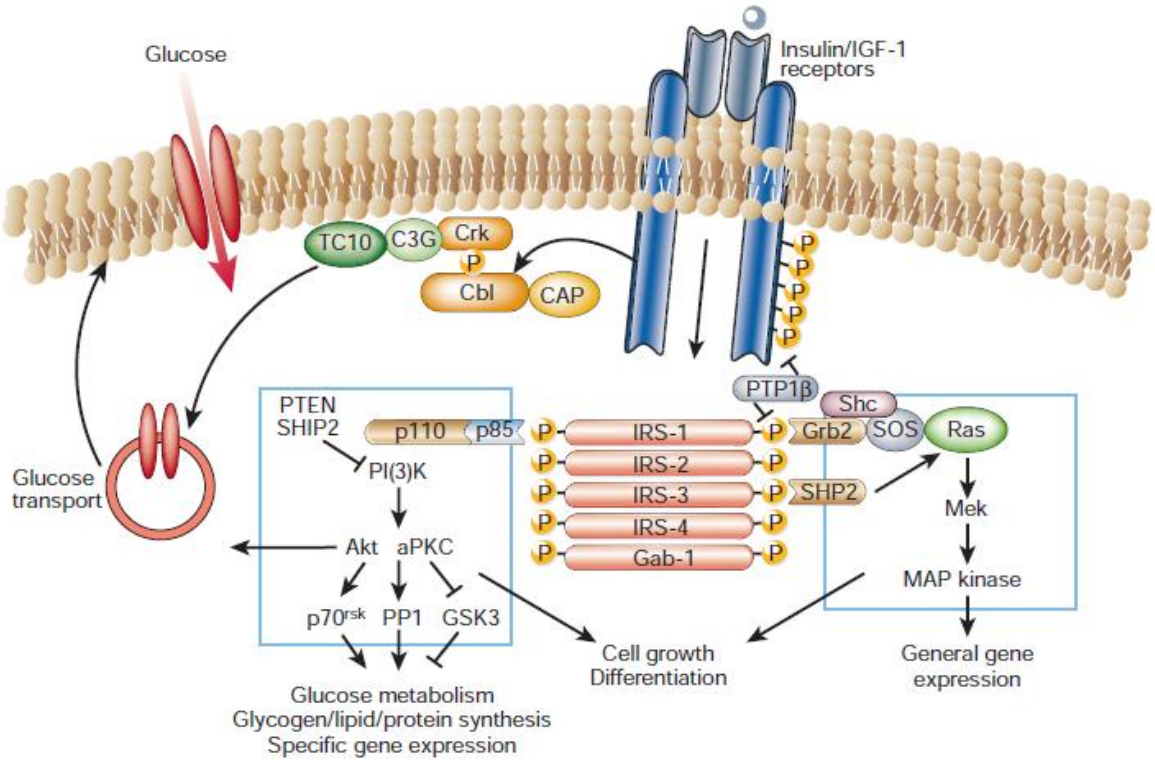


Figure 11: Signal transduction of insulin action adapted with permission from Satiel and Khan **(138)**

a) Liver

Insulin works directly via inactivation of glycogen phosphorylase (GP) which is the rate-limiting enzyme in glycogenolysis [(153)]. This action inhibits the breakdown of glycogen to glucose-6-phosphate in the liver resulting in decreased glucose output [(153)]. Phosphoenolpyruvate carboxylase (PEPCK) is an enzyme that catalyses the gluconeogenesis process from different substrates like alanine, pyruvate, amino acids, lactate and glycerol in the liver (Figure 12) [156]. The down-regulation of genes encoding for PEPCK by insulin leads to reduced expressions of genes encoding for key glycolytic enzymes fructose-1-biophosphate and glucose - 6 - phosphatase in the liver ultimately reducing glucose output into the systemic circulation [(138) (154)] (Figure 12) . In the Kerbs cycle, insulin stimulates the conversion of pyruvate to acetyl coenzyme A (Acetyl-CoA) and this process take place in the mitochondria.

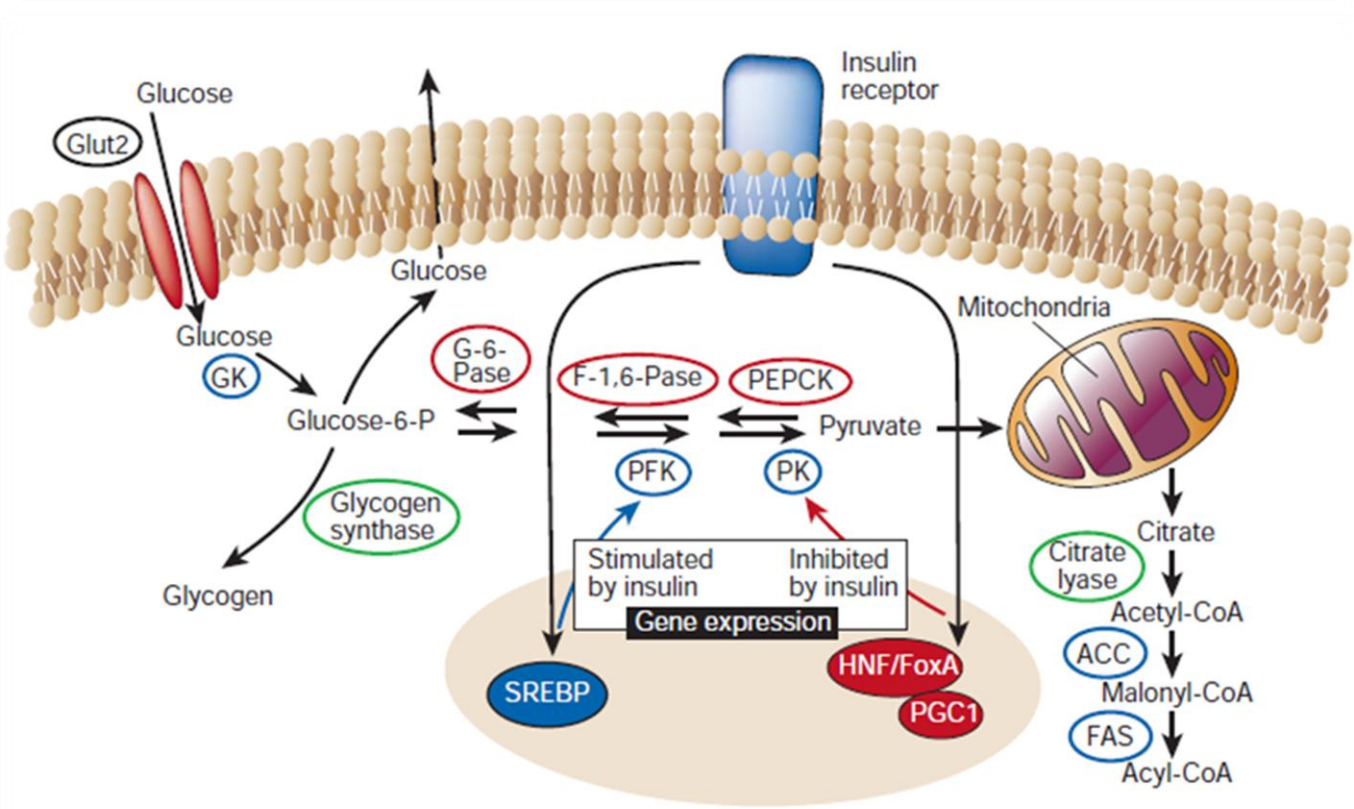


Figure 12: Hepatic regulation of glucose adapted from Saltiel and Kahn 2001 **(138)**.

b) Skeletal Muscles and Adipose Tissues

Glucose transporters (GLUTs) are specialized hexose carrier proteins that are responsible for the movement of glucose in the different tissues (155) (156). The family of GLUT's has 13 known members divided into 3 classes. Class 1 consists of GLUT 1- 4; class 2 GLUT 5,7, 9 and 11 class 3 GLUT 6,8 ,10 and 12 (155). The transport process is an ATP-independent facilitative diffusion down a concentration gradient (155) (157) (158). Insulin promotes the transport and utilization of glucose in the adipose and skeletal muscle tissues by inducing the translocation of GLUT- 4 from the intracellular site to the plasma membrane via the PI(3)k pathway resulting in protein kinase B activation and subsequent decrease in circulating glucose (119). GLUT-4 is predominantly expressed in the adipocyte and muscle tissues (156) while GLUT 2 is expressed in the liver, pancreatic β-cells liver and kidney. In the adipocyte tissues, insulin causes the stimulation of lipid synthetic enzymes such as fatty acid synthase

(FAS), Acetyl CoA carboxylase (ACC) and pyruvate dehydriogenase and the inhibition of lipolysis resulting in increased fatty acids in the blood stream [138] [144].

b) Glycolytic enzymes

1. Glucokinase

Glucokinase (GK) is a glycolytic enzyme found in the parenchyma cells of the liver and pancreatic beta cells [159]. Hexokinase I, II and III are isoforms of glucokinase and they differ in their affinity for glucose. The isoforms have a low affinity for glucose and physiological concentration of glucose 6-phosphate cannot inhibit their actions [160]. The difference between the hexokinase isoforms and glucokinase can be seen in their pharmacokinetics. When one looks at the glucose saturation curves produced by hexokinase I, II, and III, a hyperbolic curve is produced while glucokinase produces a sigmoid curve [160] [161].

Variations of glucokinase activity in the hepatic and pancreatic tissues complement each other in maintaining homeostasis [162]. In the liver it is involved in the catalytic process that promotes the phosphorylation of D-glucose to glucose -6- phosphate for utilization in the glycolytic process and other metabolic pathways and it also acts as a glucose sensor in the pancreas [159] [163] [161] . The regulation of GK gene expression is tissue specific (hepatic or pancreatic) indicative of two specific promoters on the same gene [164]. Liver GK is regulated by glucokinase regulatory protein (GKRP) which disrupts its catalytic actions [160]and in the pancreatic β-cells, glucose regulates its expression [165]. This is because the mRNA of the liver and pancreatic glucokinase differs in the 5' portion of the coding accounting for the different regulators [163]. GK governs the energy potential of the cell through oxidation and ATP generation produced during catabolism of glucose [150] [162].

$$\text{ATP} + \text{D-Glucose} \xrightarrow{\text{Glucokinase}} \text{D-Glucose-6-Phosphate} + \text{ADP}$$

2. Glycogen Synthase

Glycogen synthase (GS) is an enzyme located in the liver and muscle tissues and plays a pivotal role in glycogen synthesis. GS catalyses the addition of UDP- glucose to a non-reducing end of a glycogen chain through α-1, 4-glycosidic bonds [166] [167] [168]. GS only catalyses the bonding of UDP-glucose to an already existing glycogen chain with 4 sub units while de-novo glycogen synthesis is catalysed by glycogenin which is a glycosyltransferase. Autoglycosylation results in the formation of short glucose polymers that will be ready for elongation through the action of glycogen synthase [169]. Glycogen synthase regulation by phosphorylation, which causes a reduction in its activity and allosterocally by glucose -6- phosphate concentrations [170] [171]. Insulin causes the phosphorylation of tyrosine-kinase resulting in increased expression of glycogen phosphorylase phosphate and a subsequent increase in glycogen synthase – phosphate activity increasing glycogen formation [171].

1.7.5. Insulin Counter Regulatory Hormones

The effects of insulin can be countered by hormonal and neural regulation in response to hypoglycaemia. Glucagon, cortisol, catecholamines (epinephrine, norepinephrine) and growth hormone are antagonistic to insulin actions [172]. Glucagon is a catabolic peptide hormone that is secreted in pulses from the pancreatic α-cells in response to hypoglycaemia [173]. It antagonises insulin's actions by promoting glycogenolysis and gluconeogenesis and inhibiting the glyconeogenic processes causing an increase in the blood glucose levels. Glucagon secretion is controlled by the α-cells due to its sensitivity to high glucose levels mediated via both the parasympathetic and sympathetic divisions of the nervous system (sympathoadrenal axis and intra –islet insulin) [146] [173]. The autonomic nervous system action due to low blood glucose causes glucagon to be released through the activation of β2- adrenoreceptors by adrenaline released from the adrenal medulla [174] . The glucagon receptor is a G-protein coupled receptor and the activation of the two classes $G_S\alpha$ and G_q are an integral part of the signal transduction processes [168]. Activation of $G_S\alpha$ causes increased cellular cAMP levels through the actions of adenylate cyclase resulting in the activation of protein kinase A (PKA). PKA phosphorylates glycogen phospharylase kinase that results in glycogen breakdown through the action of

glycogen phospharylase [170]. On the other hand, the G_q activation results in increased intracellular Ca^{2+} levels subsequently decreasing glycolysis [175]. Other pathways activated include protein kinase C (PKC) and mitogen-activated protein kinases (MAPK) that are pro gluconeogenic [175]. Glycogen down regulates the gene expression of pyruvate kinase a key enzyme in the glycolysis cycle resulting in increased glucose production.

1.8.0. Consequences of Chronic Hyperglycaemia

Persistent hyperglycaemia contributes significantly to the development of diabetic complications. Several molecular pathways have been proposed in an effort to explain the pathological developments of these complications [176]. The biochemical pathways involved include the hexosamine pathway, polyol pathway, protein kinase C activation, enhanced oxidative pathways, formation of advanced glycation end products and activation of the renin-angiotensin system [177]. Accumulation and formation and or expression of diacylglycerol (DAG), protein kinase C (PKC) [178], Nuclear Factor -κB (NF-κB) [177] and reactive oxygen species generation [178] play a major role in the development of diabetic complications. Toxic metabolites produced from these pathways have been known to cause altered gene expressions; protein functions and cell activating molecules resulting in cell damage.

1.8.1. Advanced Glycation End Products (AGEs)

AGEs are formed by a non-enzymatic process in which glucose (a reducing sugar) reacts with an amine residue on a protein, lipid or nucleic acids to form schiff bases, early glycation products and amadori which are reversible intermediate glycation products [179]. These products undergo further glycation and oxidation resulting in irreversible cross linked macroprotein known as Advanced Glycated end-products [177] [180] [181] (Figure 13) . The AGEs formed from the Amadori (1-amino-1-deoxyketose) products can undergo glycation together with oxidation forming N^{ε}–[carboxymethyl]-lysine (CML), N^{ε}-carboxyethyllysine (CEL) and pentosidine or glycation without oxidation forming pyralline [179] [182].The products formed from the polyol pathway namely; 3-deoxyglucosone (3-DG), methylglyoxal and glyoxal cause an increased carbonyl stress due to their accumulation and their reaction with certain proteins like lysine and arginine or lipids forming CML a major AGE [183]. AGE formation is faster

when the reaction takes place between intracellular sugars like glucose-6-phosphate, glyceraldehyde-3-phopshate [183] and fructose because glucose has a slower glycation rate [179]. The cellular signalling caused by the AGE is mediated via interaction with receptor for advanced glycated end products (RAGE) and RAGE/Lactoferrin like polypeptide (LF-L) complex resulting in the activation of different signalling cascades [181].

RAGE is a receptor from the immunoglobulin super-family with 3 immunoglobuluin like domains located in different tissues [179] [181] [184] [185]. NF-κB, interferon –γ-response element and interleukin-6 has been identified on the RAGE promoter [183]. Accumulation of AGE and its interaction with RAGE has been linked to the development of vascular complications of hyperglycaemia [179]. RAGE stimulation has been associated with their constant activation/upregulation [184]. RAGE ligand – RAGE complex has been linked with beta cell dysfunction in type 1 and type 2 diabetes [158]. AGE causes the up-regulation of RAGE and generation of ROS via the NAD P H oxidase and this in turn causes generation of more AGEs [177]. AGE activates various signalling cascades like; MAP kinase, PKC and transcriptional factors, which cause cellular dysfunction. AGE can be detected in the serum and glomerular tissues and increased levels in these locations are strong indicators of tissue damage (Figure 14) [49] [176] [185].

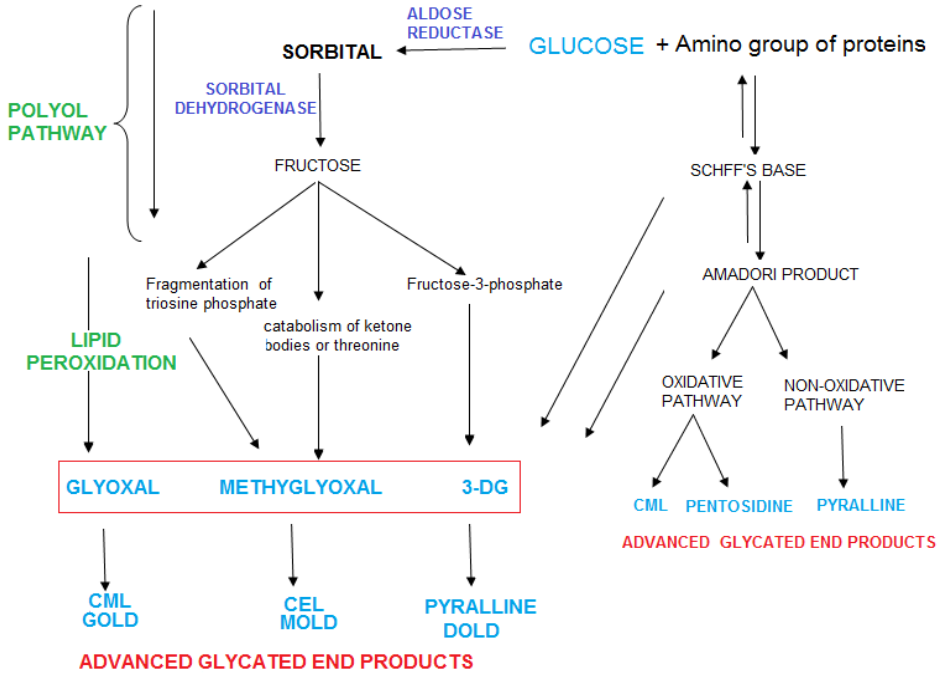


Figure 13: AGE generation form different pathways adapted and modified with permission from Singh *et al* 2004 **[179]**.

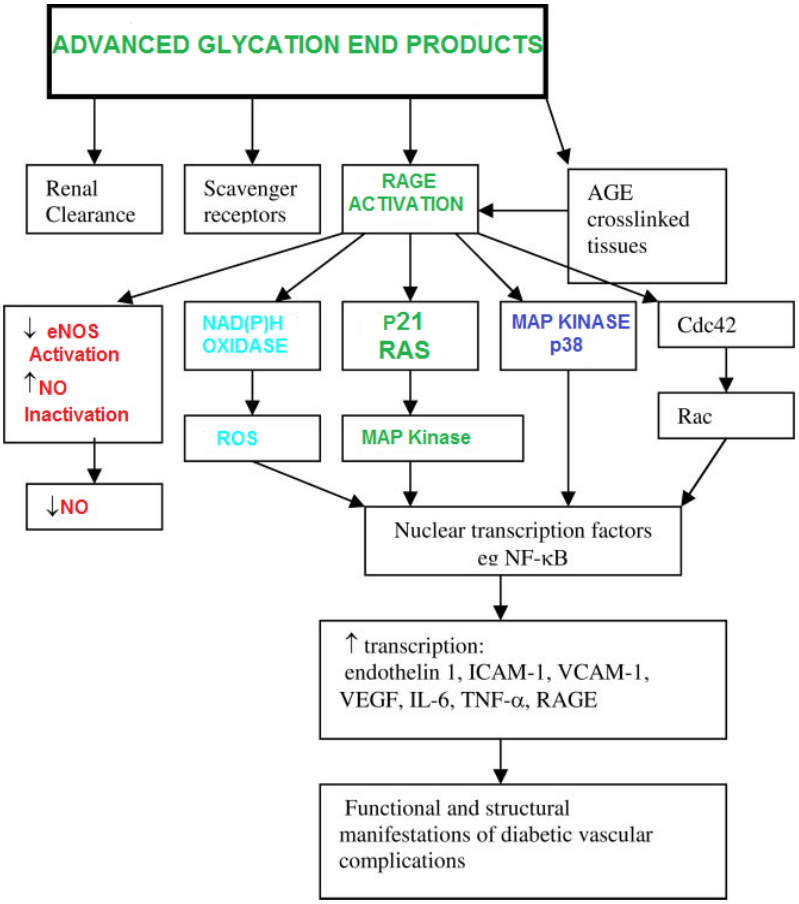


Figure 14: Signalling cascade of age mediated effects in diabetic microvasular complications adapted from GOH and Cooper 2008 **(180)**.

1.8.2 The Polyol Pathway

The polyol pathway has also been implicated in the formation of advanced glycation end products, activation of PKC and it is also a major source of ROS [(182)]. During hyperglycemia, glucose is metabolised extensively through the polyol pathway [(186)]. Aldose reductase catalyses the conversion of glucose to sorbital and it is the rate limiting enzyme in the polyol pathway [(187)]. Sorbital undergoes oxidation catalysed by the enzyme sorbital dehydrogenase (SDH) to form fructose [(176)]. Through the polyol pathway the AGE products formed are; glyoxal, methlyglyoxal (MGO) and 3-deoxyglucosone (3-DG) and they are α-oxoaldehydes [(179)] (Figure 13). In addition to formation of AGE, increased concentration of aldose reductase has been associated with the development of diabetic neuropathy and inhibition of this enzyme by zenarestat ameliorated the situation [(188)]. Accumulation of sorbital in the tissues has been associated with the reduced myoinositol and a decrease in the Na^+, K^+ activity [(189)]. Its accumulation in retinal and or vascular tissues has been associated with the

development and progression of diabetic complications like cataracts, retinopathy and aortic disease.

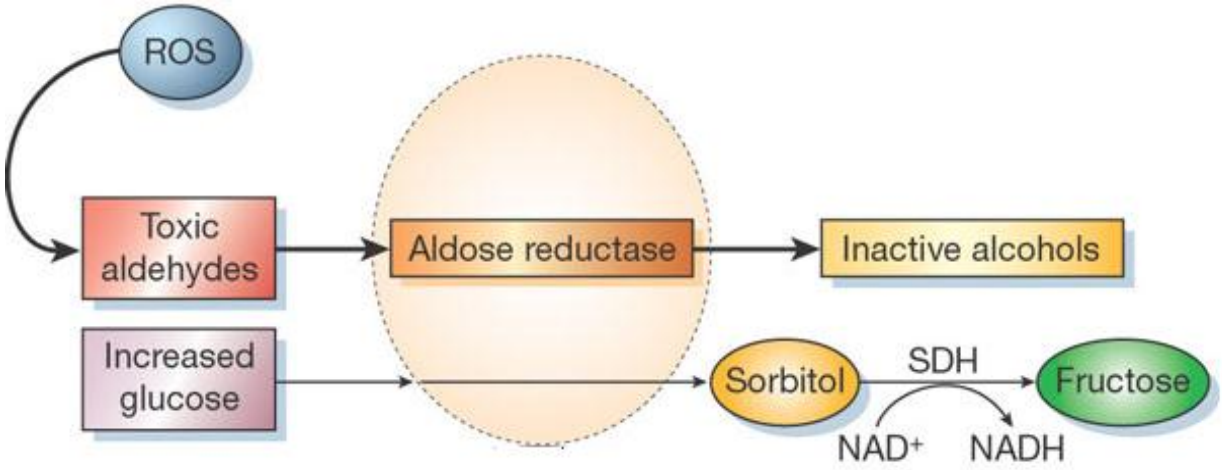


Figure 15: Sorbital production in the polyol pathway adapted with permission from Brownlee 2001 **(190)**.

1.8.3. Oxidative stress

Oxidative stress occurs when a cell is chronically exposed to reactive oxygen species [(191)]. Reactive oxygen species (ROS) is a term generally used to describe free oxygen radicals and non-radical oxidizing agents [(192).] Disruption of the redox homeostasis process leads to excessive generation of ROS leading to an increase in oxidative stress. Factors that upset the negative feedback mechanism of the body that is responsible for regulating ROS generation cause increased generation ROS. These factors include mitochondrial dysfunction, AGE formation and increased flux in the polyol pathway. Glutathione peroxidise-1 located in the mitochondrion is responsible for the breakdown of hydrogen peroxide to water. In a dysfunctional mitochondrion, hydrogen peroxide is converted to a hydroxyl radical and coupled to an impaired electron transport chain leading to increased production in ROS [(193)]. ROS generation from various organs/tissues has been associated with decline in the pancreatic β-cell function as observed in type 2 diabetes [(194)]. Glucose -6-phosphate dehydrogenase (G6PD) is the major source of nicotinamide adenine dinucleotide phosphate (NADPH) that is responsible for the activation and action of the body's enzymatic antioxidant systems [(142)]. NADPH is an important component in the functioning of glutathione reductase activity [(187)]. Zhang *et al.* [(142)] showed that hyperglycaemia

causes decreased GSH and ultimately the inhibition G6PD causing increased oxidative stress. The balance between ROS generation and stable oxygen species are maintained by enzymatic and non-enzymatic antioxidant systems produced in the body coupled with consumption of fruits and vegetables that contain flavonoids, which are strong antioxidants [53] .

The redox homeostatic balance can upset the natural antioxidant defence mechanism due to disease states. Enzymatic and non-enzymatic processes have been identified in the formation of these reactive oxygen species [192]. Kojda and Harrison in their review identified 9 enzymatic sources of ROS [191]. Some of the major sources of ROS are superoxide released from mitochondria, "leakage" in mitochondrial electron transport chain (METC) [195], membrane bound NADPH oxidase [196] and hyperglycaemia induced changes like modified polyol pathway [196] [197]. The polyol pathway causes the generation of ROS through the actions of aldose reductase a monomeric oxidoreductase and sorbital dehydrogenase [Figure 15]. The conversion of glucose to sorbital leads to the depletion of NADPH and the oxidation of sorbital leads conversion of NAD^+ to NADH [186].

NADPH oxidase catalyses the conversion of NADPH to ROS [197]. There is increasing evidence that shows NADPH oxidase plays a major role in the pathophysiology of vascular diseases, hypertension, hypercholesterolemia and atherosclerosis [197] . An increase in the NADH/NAD^+ ratio causes a decrease in nitric oxide (NO) and an alteration in the cellular redox, oxidant and osmotic stress [198]. Polyunsaturated fatty acids undergo oxidation by oxoferryl species and lead to the formation of peroxyl radicals that can cause the oxidation of membrane lipids and proteins leading to the formation of more reactive radicals [192] [195] causing an increase in oxidative stress through AGEs formation [Figure14].

Activation of transcriptional factors, for example NF-κB by RAGE-AGE interaction leads to addition of oxidative pressure on the tissues [177]. A study done by *Inoguchi et al.* [197] showed that the generation of ROS in renal mesangial cells and vascular tissues can be attributed to the activation NAD(P) H oxidase by PKC [197].

1.8.4. Protein Kinase C Pathways (PKC)

Protein kinase C (PKC) is a single polypeptide chain containing a regulatory and a catalytic region in its structure located in the N-terminal and C-terminal respectively [(199)] and is a member of the AGC family of protein kinases [(200)]. The four domains C1-C4 are binding sites for different substrates namely C1- Diacylglycerol / phorbol esters, C2- anionic lipids in a calcium dependent manner; C3 and C4- ATP and substrate binding lobes of kinase cores. The 11 iso-enzymes identified are categorised into 3 groups namely the conventional, novel and atypical according to what activates the iso-enzymes [(201)]. The different isoforms of PKC are α, β1, β2, γ, μ, ε, δ, ζ, θ, λ, ŋ and they are specific to particular cells [(178) (199) (200)]. The isoforms PKC β1 and β2 are obtained from the 47splicing of PKC β gene and it has been shown that these two isoform have antagonistic effects. The expression of PKC β1 and β2 in monocytes is regulated by insulin in a dose dependent manner by controlling the splicing of the parent gene and the expression of PKC β subtypes [(202)]. PKC isoforms α, β1 and ζ are increased in glomerular tissues of diabetic rats, α, β2 and ε in the retinal tissues accounting for the changes associated with the various organs [(203)].

Calpains a cytosolic calcium-dependent cysteine protease has also been implicated in diabetic vascular dysfunction. A study done by Smolock *et al.* [(202)] revealed that the PKC isoform (PKC β) causes the activation of endothelial expressed calpain which causes an increase in cell adhesion molecule (ICAM-1), causing an increased leukocyte-endothelium interactions which have been implicated in vascular complications [(198)]. An increase in the NADH: NAD^+ ratio from increased flux in the polyol pathway causes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to be inhibited. Its inhibition results in the increase of triose phosphate and consequently the formation of methylglyoxal an AGE precursor and diacylglyceol (DAG) [(190)]. An increase in Ca^{2+} and DAG a lipid second messenger causes the activation of the PKC pathway [(203)]. The formation of DAG is dependent on the actions of Phospholipase C. This enzyme is located in mammalian tissues and is responsible for hydrolysis of inositol phospholipids resulting in the formation of DAG and inositol phosphates [(204)].

The PKC signal cascade can starts from the reduction of dihydroxyacetone phosphate a glycolytic intermediate to glycerol-3-phosphate a precursor for *de novo* synthesis of DAG [201]. *In vivo* and *in vitro* studies show that chronic hyperglycaemia creates an internal environment that is conducive for DAG formation leading to sustained activation of PKC resulting in vascular complications [176] [197]. The activation of PKC via different pathways causes specific changes in various tissues and alteration of function in certain enzymes. PKC activation causes an increases in tissue permeability, expansion of extracellular matrix, thickening of the basement membrane, alteration of mitogen activated protein kinase function (MAPK), $NA^{+}K^{+}$-ATPase and transcription factors [201]. Endothelial dysfunction is accompanied by a decreased production of prostacyclin, nitric oxide (NO) that is responsible for maintaining blood vessel integrity and an increase in endothelin-1, vascular endothelial growth factor and cyclooxygenase dependent vasoconstrictors [198] [201]. The inhibition of the DAG-PKC pathway by PKC specific isoform inhibitor ruboxistaurin mesylate improves haemodynamic changes in nephropathy, retinopathy and neuropathy [205] thus ameliorating the detrimental effects mediated by the activation and action of Protein Kinase C.

1.9.0. Diabetic complications

Metabolic and haemodynamic factors intertwined together in diabetic patients are associated with the development of cardiovascular diseases, stroke, atherosclerosis, nephropathy, neuropathy and retinopathy [206]. These complications can be categorised into two major groups namely macrovascular and microvascular complications.

1.9.1 Microvascular Complications

1.9.1.1. Diabetic Neuropathy

Diabetic neuropathy is a disorder of the peripheral nervous and autonomic nervous system as well as some cranial nerves due to chronic effects of hyperglycaemia [207]. This neurological complication affects both type 1 and type 2 diabetic patients with long standing diabetes [188]. Diabetic neuropathy is the leading cause of non-traumatic amputations, ulcers that take long to heal and nerve injury [176]. There are different types of neuropathies and they are be classified as symmetric and asymmetric with their clinical manifestations dependent on the group of nerves affected. The aetiology

of diabetic neuropathy is not well understood but it is believed that, the formation of AGE, increased oxidative stress, increased polyol pathway and diabetic vascular disease play a pivotal role in the progression of the disease [188]. It is characterised by segmental demyelination accompanied by loss of myelinated and unmyelinated axons [208]. It is speculated that hyperglycaemia causes the depletion of neurotrophins that are responsible for normal axon repair [188] and the progression of the complication by AGE has been shown to cause a decrease in the sensorimotor conduction velocity and reduced blood flow in peripheral tissues [156]. Treatment with aldose reductase inhibitor fidareplast improves nerve conductance velocity and palrestat can delay the progression of the disease and ameliorates the associated symptoms [176].

1.9.1.2. Diabetic Retinopathy

Retinopathy is the major cause of blindness in diabetic patients. Biochemical pathways involved in the pathophysiological development of diabetic retinopathy have since been identified. The retinal changes associated with diabetic retinopathy are haemorrhages, microaneurysms and proliferative retinopathy [207]. The earliest morphological changes that can be seen are loss of pericytes and a cellular capillary formation [188]. In the retinal tissues of diabetic subjects there is an increased accumulation of DAG and a consequent activation of PKC [176] isoform β that causes increased vascular permeability and formation of microaneurisms and the PKC δ causes retinal pericyte apoptosis through the activation of NF-κB [176] [201]. These changes cause glaucoma and cataracts and if not treaded could lead to total blindness.

1.9.2 Macrovascular Complications

1.9.2.1. Atherosclerosis

Diabetes is a major risk factor associated with the development of stroke, coronary heart disease, peripheral artery disease and atherosclerosis [211]. Atherosclerosis and thrombogenic complications are a major cause of morbidity and premature death in diabetic patients [208]. Multiple independent pathways discussed previously have been implicated in the development and pathological progression of atherosclerosis. In diabetic patients with atherosclerosis, dyslipidaemia is characterized with high levels of low density lipoprotein (LDL) and very low density lipoprotein (VLDL) and decreased high density lipoproteins (HDL) [39]. Increased levels of glycation and lipid

peroxidation products in diabetic patients especially apoprotein B and phospholipid portion of LDL have been associated with alterations of LDL clearance and oxidative modifications. AGE binding to Apo B impairs its clearance in the hepatic tissues and enhances the retention of LDL accumulation on the aortic wall resulting in an inflammatory process accompanied by the formation of atheromas [180].

Increased expression of RAGE in the human atherosclerotic plaques is co-localised with cyclooxygenase 2, microsomal prostaglandin E_2 which causes destabilization of the plaques via the action of vascular dependent matrix metalloproteinase-2 which cause excessive break down of ECM resulting in plaque rapture [209]. Pharmacological agents aminoguanidine and alagebrium have been identified as inhibitors of AGE and RAGE expression minimizing the expression of prosclerotic growth factors in experimental animals but further studies need to be conducted on them [180]. Intensive therapy with HMG CoA reductase inhibitors have proven to be beneficial agents in preventing adverse effects associated with lipid peroxidation and lipid modifications reducing the incidents of lethal cardiovascular events [210].

1.9.2.2. Diabetic Nephropathy

Diabetic nephropathy (DN) is a common microvascular complication associated with diabetes mellitus and is the leading cause of end stage renal disease globally (ESRD) [177] [184] . Epidemiological data estimates that 30-40% of patients with type 2 diabetes and 15-20% of type 1 diabetics will develop chronic renal failure [176] [211] .The risk of developing nephropathy is increased in genetically predisposed diabetic patients with uncontrolled hyperglycaemia. DN is characterised by the presence of protein in the urine, elevated blood pressure [212] and progressive renal insufficiency [189] due to glomerular lesions and loss of glomerular filtration rate in the absence of albuminuria [213]. The different stages of DN are determined by the extent of renal impairment and presence of albuminuria [214]. The uniferous system plays a fundamental role in the glucose homeostatic processes, because of sodium glucose co-transporters (SGLT) located on the renal proximal tubules (PCT) brush border that is responsible for the reabsorption of glucose from the glomerular ultra-filtrate [215] [216] [217] [218] . This hexose carrier protein is also localised in the intestinal lumen where it aids in glucose movement using sodium co-transport as an energy source. Gluconeogenesis in the

renal cortex also plays a part in increasing the body's glucose load (Figure16) [(219)] . SGLT1 and SGLT2 are expressed in different portions of the PCT and are responsible for the reabsorption of glucose coupled with sodium across a concentration gradient into the tubules. GLUT1 and GLUT 2 aid its movement across the interstitium back into the systemic circulation (Figure 17) [(219)]. The functioning of these transport proteins is augmented by the activity of Sodium/Potassium-ATPase pump on the basolateral membrane. In the diabetic state the glucose threshold in the PCT is increased from 350 mg/min (normal) to 420 mg/min coupled with increased expression of SGLT2 [(220)]. Increased expression of the SGLT2 in PCT causes a significant increase of glucose reabsorption resulting in increased hyperglycaemia that causes metabolic and haemodynamic changes which impact greatly in the pathogenesis on DN. Phytochemicals like naringenin found in many citrus fruits have shown potential as potent SGLT2 inhibitors in intestinal and renal cortical brush border membrane vesicles in rabbit and rat tissues [(80)]. This effect could prove beneficial in the regulation of blood glucose in type 2 diabetic patients but its effects in diabetic models still need to be investigated further [(122)].

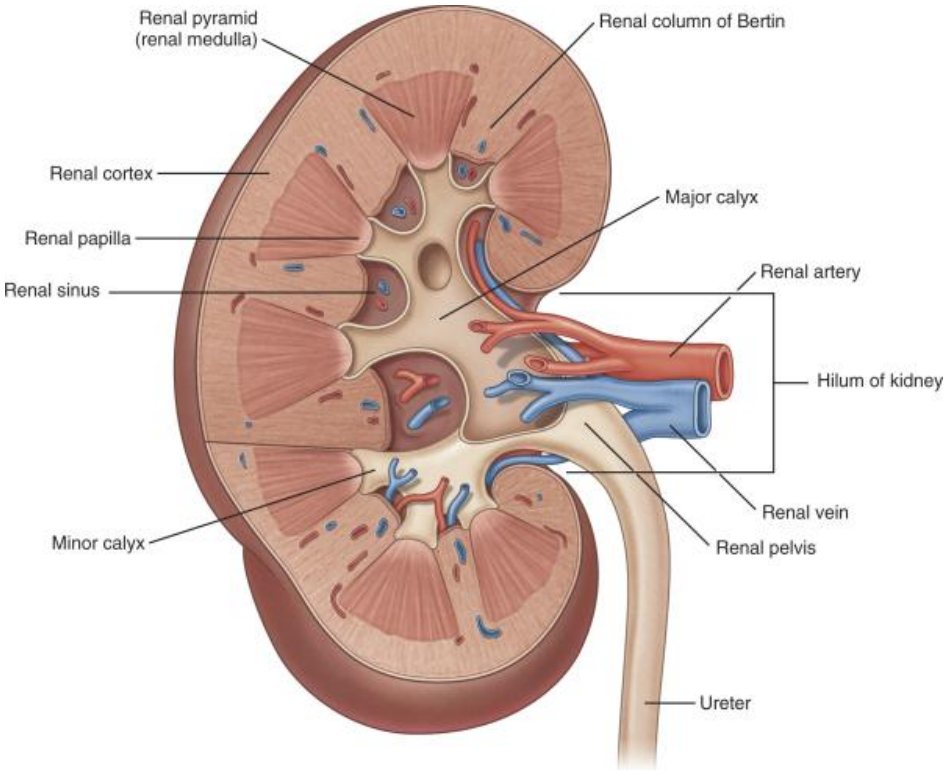


Figure 16: Saggital section of the kidney depicting the macroscopic parts cortex and medulla [218]

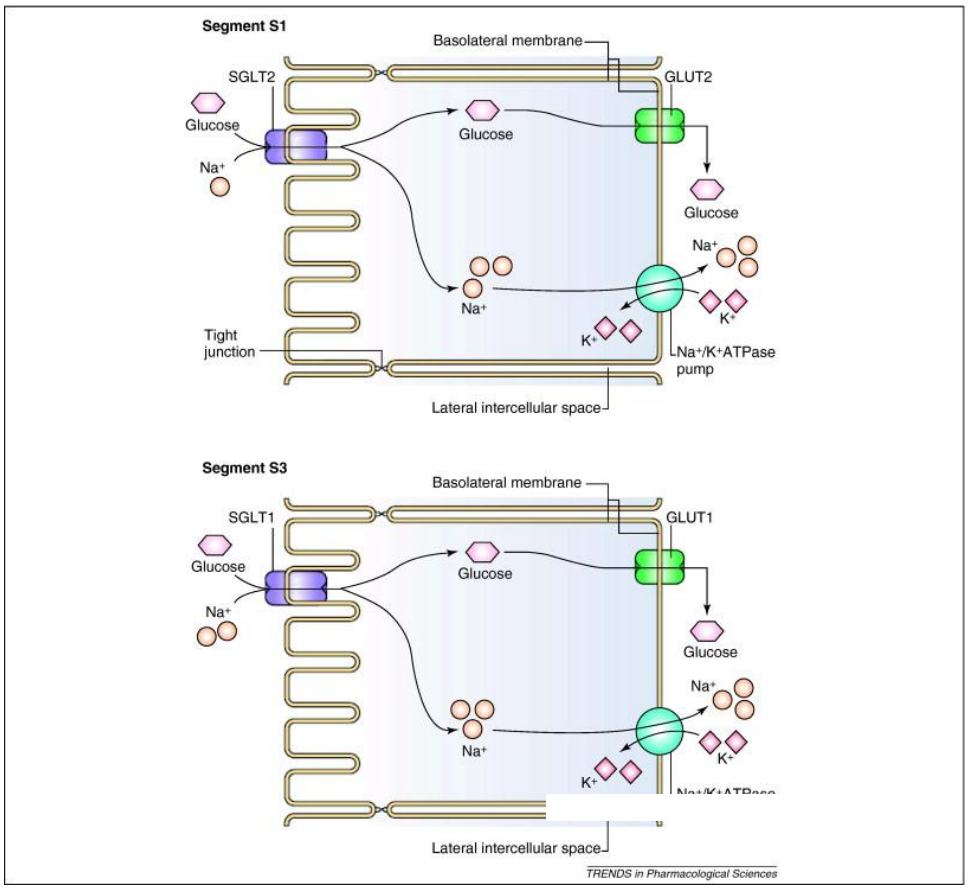


Figure 17: Glucose movement in the S1 and S3 portions of the proximal convoluted tubule adapted with permission from Bailey 2007 [(219)].

Chronic hyperglycaemia on renal tissues has detrimental effects to the normal functioning of the kidney and subsequent decline in the kidney functions. Structural changes in the renal corpuscle and surrounding tissues as a result of metabolic and haemodynamic effects induced by hyperglycaemia are implicated in development of renal injury and subsequent dysfunction. Molecules like monocyte chemoattractant protein-1(MCP-1),intracellular adhesion molecule -1(ICAM-1), cyclooxygenase 2 (COX-2) , nitric oxide synthase, vascular endothelial growth factor (VEGF), transforming growth factor β , nuclear factor κβ (NF- *κβ*) and inflammatory cytokines have been identified as contributing factors to the progression and pathogenesis of DN [(221)].

1.9.2.3. Pathological Development of Diabetic Nephropathy

In DN, thickening of the glomerular basement membrane (GBM) highlighted in green (Figure 18) is accompanied by mesangial expansion, thickening due to fibrosis and expansion of tubulo-intestitial compartments due to increased extracellular matrix deposition [198] [222]. In the progression of DN glomerular sclerosis, arteriolar hyalinosis, and tubulo-interstitial remodelling are the pathological features observed in the advanced stages [223]. Mesangial cells located in the glomerulus are responsible for the synthesis of extracellular matrix (ECM) and accumulation of the ECM is a major problem that results in alteration of the renal structures. Matrix metalloproteases (MMP) are zinc dependent endopeptidases responsible for the degradation and physiological regulation of ECM but in the diabetic state their functioning is impaired [68].

The kidney is the major site for AGE clearance and its dysfunction contributes to increased accumulation of AGE. AGE accumulation plays an active role in the impairment of the renal functions because of its direct effects on matrix metalloproteases impairing their function [177] [168] [182]. AGE [224], hyperglycaemia, reactive oxygen species and transforming growth factor β (TGF-β) [225] trigger the expression of TGF-β and connective tissue growth factor (CTGF/ CCN2) which contribute to ECM thickening. The actions of CTGF are diverse but they all contribute to the process of fibrosis by fuelling the production of the protein fibronectin and plasminogen activator inhibitor synthesis [225]. Increased expression of urinary CTGF (uCTGT) has been shown as an important biomarker in the assessment of tubular function because CTGF is fully reabsorbed in the PCT and increased uCTGF has been observed in patients with DN [226] . In addition, Cell division autoantigen 1 (CDA1) an antiproliferative, Angiotensin Converting Enzyme II (ACE II) and profibrotic growth factor similar to TGF-β also contribute to these renal changes [214]. Vascular remodelling due to increased ECM deposition results in the crushing of glomerular capillaries which in-turn compromises the glomerular filtration processes due to loss of the capillaries selective permeability.

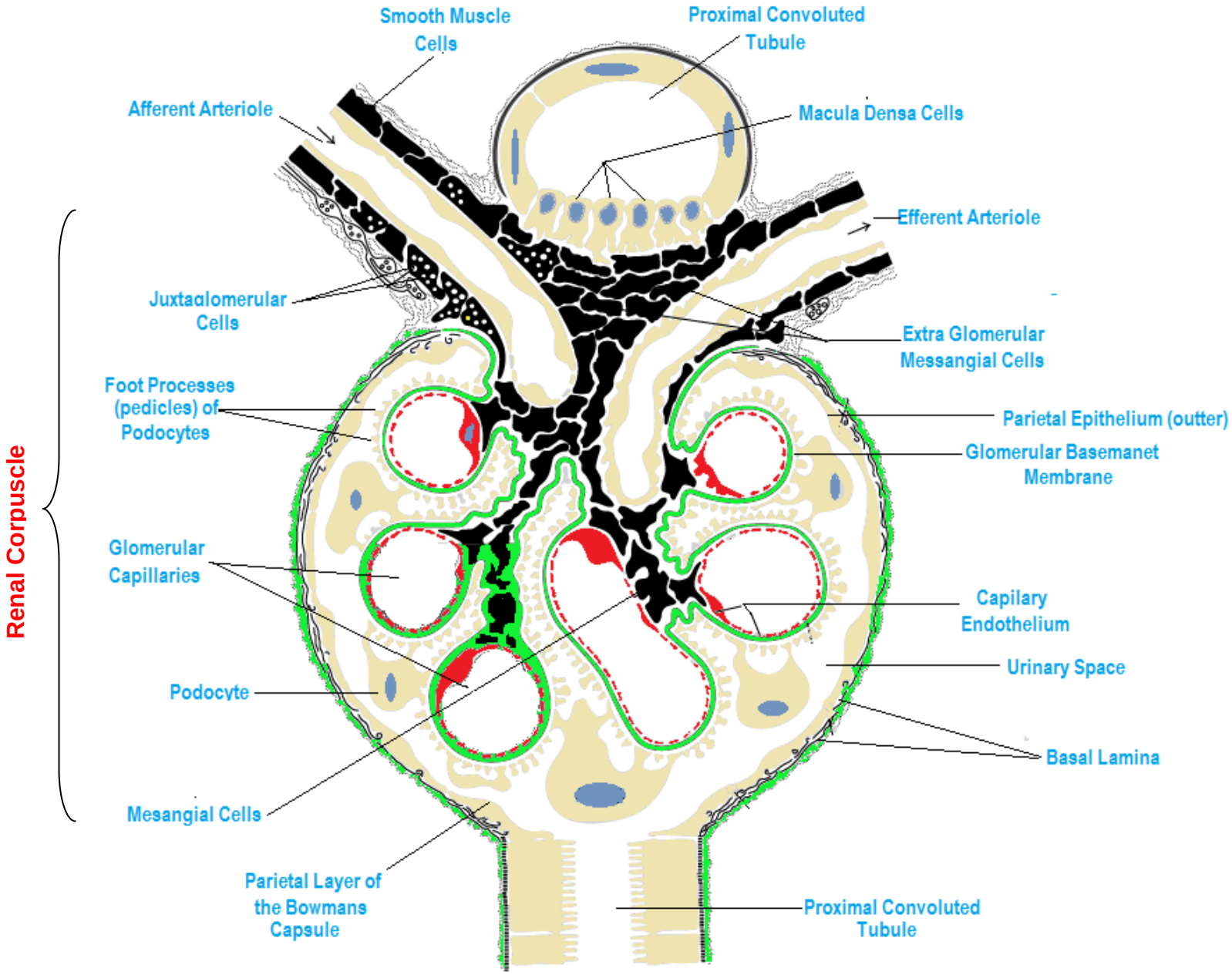


Figure 18: Schematic organization of the renal corpuscle adapted and modified with permission from Ross and Pawlina [256]

Glomerular hypertension, hyperfiltration and endothelial dysfunction are some of the haemodynamic factors that have been associated with a decrease in NO and an increase in ACE II in DN. Angiotensin converting enzyme (ACE) in the kidney catalyses the conversion of angiotensin I to angiotensin II . It has been reported that Angiotensin II plays a major role in the progression of DN by adding to the haemodynamic and structural changes [(214)]. Some of the effects of ACE II are mediated via increasing the synthesis of plasminogen activator inhibitor -1(PAI-1) [[267]], activation of transforming Growth factor β (TGF-β) and Smad3 [(227)]. Smads are cytoplasmic proteins that are substrates for type 1 TGF β receptors [(228)]. Activation of pro-sclerotic cytokines like TGF-β by hyperglycaemia at the transcriptional level, and angiotensin II advance the progression of DN by promoting matrix production and inhibiting their degradation while increasing the matrix receptors [(219)]. These changes modify the cell structure and function resulting in defective cell adhesion and altered cell growth [(229)]. Studies have confirmed an increased expression of TGF-β in renal tissues of diabetic animals and administration of TGF-β inhibitors such as CTGF-antisense oligonucleotide or CTGF-siRNA, ACE inhibitors and angiotensin receptor blockers ameliorate the detrimental effects of TGF-β thus conferring renoprotection[(68)]. Increased expression of endothelin-1, aldosterone and angiotensin II contribute to oxidative stress by increasing the activity of NADPH oxidase [(230)].

Inflammatory processes also play a major role in the development and progression of DN. The binding of AGE to its receptor (RAGE) in the renal tubules results in the formation of myofibroblasts and this process is implicated in tubular atrophy and interstitial fibrosis [(229)] . In addition, AGE/RAGE interactions cause the activation and/or expression of inflammatory mediators that contribute to vascular dysfunction [(177)] [(213)]. TNF-α has been implicated in the development of glomerular insulin resistance, reduction in the glomerular filtration rate, local generation of ROS and production of endothelin-1 resulting in organ dysfunction. Increased RAGE expression in vascular tissues of diabetic patients triggers the expression of adhesion molecules like vascular cell adhesion molecule- 1(VCAM-1), IL-6, MCP-1and generation of ROS [(182)]. This is accompanied by increased glomerular deposition of AGE on the capillaries which lead to the expression of VEGF, VEGF receptor and RAGE. VEGF expression results in increased vascular fenestrations at the glomerular endothelial brush border resulting in capillary basement modification. These fenestrations allow large molecules like albumin to pass through and be excreted in urine resulting in albuminuria, a characteristic feature in DN [(182)] Treatment with VEGF receptor -2 antibodies and soluble RAGE (sRAGE) improved

glomerular hypertrophy and albuminuria in diabetic *db*/*db* mice [182] . In addition, the expression of NF-*κ*B, a transcriptional factor located on the RAGE promoter and early growth response 1(Egr-1) have been implicated in the inflammatory processes that take place in the kidney. Increased expression of NF-*κ*B causes pro-inflammatory cytokines like IL-1 α, IL-6, TNF-α to be expressed with PKC contributing significantly to renal inflammatory processes, [179] which cause serious tubular inflammation and cell proliferation that contribute to the development and progression of DN [230].

Angiotensin II, K^+ and adrenocorticotropic hormone (ACTH) stimulate the expression of aldosterone which plays a central role in regulating Na^+, K^+ , Cl^- and water balance in the renal tubules. Aldosterone works by stimulating Na^+ absorption via Na^+/ K^+-ATPAse which is accompanied by Cl^- reabsoption and K^+ excretion [231]. Increased expression of aldosterone is characterised by increased inflammation and matrix accumulation [230]. Mineralcorticoid receptors on the glomerular mesangial cells mediate the actions of aldosterone and this causes proliferation and increase in ROS generation in SGK1 [230]. *In vivo* and *in vitro* results hae shown that treatment with spirolactone down-regulates the expression of VEGF, insulin growth factor (IGF) and TGF-β, thus improving renal function [230].

In the late stages of DN, reduced GFR is accompanied by increased Na^+ retention in patients suffering from diabetes mellitus. Na^+ retention can plays a major role in the development of arterial hypertension [232]. Altered ion transport in the PCT as a result of hypertrophy due to effects of CTGF and tubular atrophy play a major role on DN progression. IGF-1 has been implicated in the increased retention of Na^+ in diabetic patients. Its receptors are located in the glomerulus and tubular segments in the apical and basolateral membranes. Vascular permeability due to increased fenestrations causes the IGF-I from the plasma to be filtered through the glomerulus. The ultrafiltrate containing IGF-I enhance Na^+ transport in the distal convoluted tubule by binding to apical membranes enhancing Na^+ transport [233].

In addition elevated levels of insulin have been known to cause stimulation of sodium reabsorption in the DCT due to its antinatriuresis effects [234]. The reabsorption of Na^+ along the nephrons is controlled by different regulators and inhibition of these regulators can result in altered sodium concentrations (Table 8). Elevated plasma levels of D-glucose and or IL-1β causes an increase in hyaluronic acid (HA) in injured proximal tubule. HA has been known to induce the up-regulation of ICAM-1, VCAM-1 and chemoattractant protein 1 expression

which contributes to the progression of diabetic nephropathy [235] [236]. In the tubular endothelium *myo-inositol oxygenase (*MIOX) is responsible for the oxidation of Myo-inositol that is generated from Glucose -6- phosphate. Its metabolic product phosphatidylinositol has osmoregulatory function in the kidney. Up regulation of MIOX in diabetic animals and supplementation normalizes "glucose-induced proliferation and collagen synthesis in tubular cells" [198].

Tubule segment	**% of Na^+ Reabsorbed**	**Mode of Reabsorption**	**Regulatory factors**
Proximal Tubule	50-55%	Na^+/H^+exchanger Na^+-Co-transport	Angiotensin II, GFR Norepinephrine
Loop of Henle	35-40%	Na^+/K^+ $2Cl^-$ Co-transport	Flow rate
Distal tubule	5-8%	Na^+/Cl^- Co-transport	Flow rate
Collecting tubule	2-3%	Na^+-Channels	Aldosterone , Atrial Natriuretic peptide

Table 8: Percentage of Na^+ reabsorbed in the renal tubules

1.9.3. Diabetes Management

The current approach used in the management of type 2 diabetes mellitus involves the adoption of a healthy lifestyle which includes weight control, physical exercise, and proper nutrition in combination with pharmacological therapy in order to prevent the development and or progression of micro and macro-vascular complications associated with poor glycaemia control [237].

1.9.4. Pharmacotherapy of Diabetes

1.9.4.1 Insulin Therapy

Type 1 diabetes is managed by the use of exogenous insulin to lower the blood glucose levels and to prevent development of diabetic ketoacidosis (DKA) [78] [110] [111]. There are 4 main types of insulin available in the market today i.e.: rapid acting with fast onset but short duration (Insulin lispro and aspart), short acting with rapid onset of action (humulin and novolin R, L and N) intermediate acting (Lente insulin, glargine and NPH insulin) and long acting (ultralente humulin and insulin glargine- lantus) [238]. Insulin therapy can also be introduced to patients with type 2 diabetes who are already on oral hypoglycaemic agents. The addition of insulin to the treatment regimen helps to lower the blood glucose concentration and reduce adverse effects associated with chronic hyperglycaemia. There are limitations associated with the use of exogenous insulin. One of the major problems is the high incidence of iatrogenic hypoglycaemia associated with their use. At least 90 % of type 1 diabetics have experienced a hypoglycaemic episode and this can be fatal [172] [239]. The other dilemma is pain associated with repeated injections at the same site and improper storage that could lead to insulin degradation and instability [240]. In addition to hypoglycaemia and pain, weight gain due to reduced calorie leakage into urine and increased appetite can make patients skip dosages with the resultant effect of poor glycaemia control [241].

1.9.4.2 Oral Hypoglycaemic

In type 2 diabetes, the pancreatic β-cell is dysfunctional but can still produce insulin that is not sufficient to maintain euglycaemia. Pharmacological management is by the use of oral hypoglycaemic agents and in severe cases in combination with exogenous insulin [242]. There are 5 main classes of oral hypoglycaemic agents available in the market (Table 9). They can be categorised as insulin sensitizers, insulin secretagogues and inctretin based therapies.

a) Insulin secretagogues

Meglitidines, sulfonylurea and D-phenyl alnanine derivatives increase the secretion of insulin from the pancreatic β-cells. This is because the sulfonylurea receptor is located on the ATP sensitive K^+ channel on the pancreatic β-cell. Binding of sulfonylurea to the sulfonylurea receptor -1(SUR-1) causes decreased influx of K^+ leading to depolarization of the voltage sensitive calcium channel resulting in insulin secretion [(200)]. Meglitidines on the other hand are non-sulfonylureas but act in a similar manner to sulfonylureas. The problem associated with their use is the incidence of hypoglycaemia, increased cardiac arrhythmias because of its activity on SUR2 in vascular and cardiac tissues [(116)]. The use of sulfonylureas alone is not sufficient to maintain the desired glycaemic control and a second hypoglycaemic agent sometimes is required [(243)]. Secondary failure associated with the use of sulfonylureas can be due to reduced glomerular filtration rate casing an increased risk of hypoglycaemia, insulin resistance [(238)] and β-cell destruction leading to decreased insulin production and consequently therapy failure.

b) Insulin sensitizers

Metformin is a biguanide that has been used since the 1950s to manage hyperglycaemia and it is currently the first line treatment for type 2 diabetes. Metformin improves insulin sensitivity in hepatic and peripheral tissues and is beneficial in patients suffering from IR [(228)]. In the skeletal muscles it causes increased translocation of GLUT-4 and the activity of insulin receptor tyrosine kinase this causes an increase in glucose uptake [(228) (243)]. In addition, it stimulates glycolysis and reduces the gluconeogenesis process in the skeletal and hepatic tissues [(238)]. Though it manages to lower blood glucose levels significantly, it causes minor gastrointestinal disturbances (diarrhoea, nausea, vomiting and abdominal discomfort) and lactic acidosis [(135) (144)]. Metformin is currently contraindicated in diabetic patients suffering from renal disease /dysfunction, cardiac insufficiency, DKA because of the increased risk of developing lactic acidosis [(238)].

Thizolidinediones work by binding to the nuclear transcription factors peroxisome proliferator activated receptor γ (PPARγ). They improve insulin sensitivity in the peripheral tissues by promoting the uptake of free fatty acids in subcutaneous adipose tissue [(238)] . The binding with the PPARγ causes increased expression of GLUT2 and GLUT4 and subsequent increase in glucose uptake from the circulatory system [(228)]. They reduce plasma triglyceride

levels and increase HDL – cholesterol and LDL- levels with increased LDL- levels being an undesirable effect [243]. The adverse effects associated with their use are weight gain, haemodilution, increased risk of cardiac failure and fluid retention [116].

c) α-glucosidase Inhibitors

α–amylase and α–glucosidase are intestinal brush border enzymes that metabolize carbohydrates (oligosaccharides and polysaccharides) [102] [228]. α- glucosidase inhibitors work by preventing the breakdown of complex carbohydrates by inhibition of the α- glucosidase enzymes reducing the entry of glucose into the systemic circulation thus preventing post prandial hyperglycaemia (PPH). The side effects associated with their use is diarrhoea, bloating and flatulence [228] [238]. The advantage of α- glucosidase inhibitors use is that the incidence of hypoglycaemia and weight gain are greatly reduced [243].

d) Incretin Based therapy

Glucagon like peptide -1(GLP-1) and glucose dependent insulinotropic peptide (GIP) are the major incretins produced by the body in response to oral administration of food [148] [228] [244]. The protease dipeptidyl-peptidase IV (DPP-$_4$) acts on the GLP-1 degrading it after a short period rendering it pharmacologically inert but the development of DPP-$_4$ inhibitors (Sitaglipin) increases GLP-1's half-life rendering it useful in the management of hyperglycaemia [244]. The production of GLP-1 mimetics (Exenatide) proved to be a great success because of increased half-life and ultimately improved pharmacologic properties [148] [244] [245]. GLP-1 stimulates the release of insulin from the pancreatic β-cells. This stimulation is dependent on the physiological concentration of glucose. This effect is important because it helps reduce the incidences of hypoglycaemia [245] and the inhibition of glucagon secretion and gastric emptying delay helps reduce incidences of postprandial hyperglycaemia [228]. Incretin based therapy is slowly gaining popularity in clinical practice because of its glucose dependent insulin secretion and inhibition of glucagon secretion. Other agents that are still undergoing clinical trials (stage III) include SGLT2 inhibitors such as dapagliflozin, remogliflozin, etabonate, sergliflozin and AVE2268. These agents inhibit sodium –glucose co-transporters responsible for re-absorption of glucose in the renal tissues and researchers hope that they will not be associated with the draw backs associated with the current pharmacological therapies [246].

CLASS	EXAMPLE	ACTION
Biguanides	Metformin	Reduced hepatic glucose output
Sulfonylureas	Acetohexamide*, Tolazamide*Chlorpropamide*, tolbutmide*. Glimepiride**, Glipizide , gilbenclamide**	Increase insulin secretion
Meglitidines	Repaglinide, Nateglinide	Increase insulin secretion
α-glucosidase inhibitiors (AGIs)	Acarbose, Miglitol, voglibose	Inhibits intestinal digestion Of polysaccharides
Thiazolidinedines (TZD)	Pioglitazone, Rosiglitazone,	Express transcription factors promoting insulin sensitivity, secretion
Incretins and its Analogues	Pamlitide, Exenatide, Liraglutide	Enhances post-prandial insulin secretion

1st generation *, 2nd generation**

Table 9: Classes of oral anti-diabetic agents

The number of hypoglycaemic agents has increased significantly over the years with development and discovery of new agents. The complex pathophysiology and co-morbidities associated with type 2 diabetes makes monotherapy with the available agents ineffective [(122)]. To manage hyperglycaemia and the related complications, multiple therapeutic agents are used and this can be very costly and cumbersome because of the associated side effects. Insulin sensitizer's metformin and TZDs manage to reduce the risk factors associated with development of cardiovascular diseases in diabetic patients but the use of metformin is contraindicated in patients with renal insufficiency [(247)]. The management of diabetic nephropathy currently is limited to therapies that block the rennin-angiotensin - aldosterone-system (RAAS) [(212)]. ACE inhibitors, Angiotensin receptor blockers (ARB) and aldosterone antagonists are antihypertensive agents that work on the RAAS conferring renoprotection by helping prevent the initiation of diabetic nephropathy in type 1 and type 2 diabetic patients [(212)]. In some cases where the disease has progressed ARB or ACE inhibitors do not slow down or prevent the development of ESRD requiring other interventions. Other agents under investigation are AGE/RAGE inhibitors, PKC inhibitors and TGF-β inhibitors are in progress but their safety and efficacy in diabetic patients is yet to be established. The complex pathophysiology and progressive nature of diabetes and its related complications has called

for new treatment interventions that would help prevent progression of DN and development of ESRD.

1.9.5. Basis of Study

Grapefruit in particular has gained much recognition because of its beneficial medicinal effects and weight loss properties. Recent studies have shown that the consumption of GFJ helps to improve blood glucose levels, ameliorates diabetic dyslipidaemia [39], damage caused by free radicals in diabetic tissues [38] [46] and helps improve metabolic syndrome [25]. Evidence from our laboratory shows that STZ-induced diabetic rats and non-diabetic rats treated with GFJ experienced increased polydipsia suggesting increased salt-water excretion by the kidney [39]. Naringin a bioactive component in GFJ inhibits glucose transporters in the renal tissues [80] while naringenin possesses anti-inflammatory and anti-fibrotic effects in diabetic mice [234]. GFJ has a high concentration of naringin but very little is known about its effect on fluid and electrolyte homeostasis. This study was aimed at investigating the effect of GFJ on renal dysfunction and hyperglycaemia in STZ-induced diabetic male wistar rats.

1.9.5.1 Hypothesis

Ruby Grapefruit juice (*Citrus Paradisi* MACF., FAMILY Rutacaeae) possesses anti-hyperglycaemic and renoprotective effects in STZ-induced diabetic rats.

1.9.5.2. Aims and objectives

Aims

The aim of this study was to investigate the effect of GFJ on glucose homeostasis and renal electrolyte and water balance in STZ-induced diabetic rats.

Objectives

1. To investigate the effects of chronic administration of GFJ on glucose intolerance in STZ- induced diabetic rats.
2. To investigate the effect of GFJ on electrolyte and water balance in STZ-induced diabetic rats.

Chapter 2

Materials and Methods

2.0. Materials

Chemical Reagents

The chemical reagents used in this study were purchased from Sigma –Aldrich Pty. LTD., Johannesburg South Africa are: D-glucose, streptozotocin, citrate ($C_6H_8O_7$), phosphate buffers, glycogen from oyster, sulphuric acid (H_2SO_4), potassium hydroxide (KOH), ethanol, phenol and sodium sulphate, haemotoxylin, potassium alum, citric acid, chloral hydrate ,sodium Iodate, concentrated hydrochloric acid (HCl), celestine blue B, ferric ammonium sulphate, glycerin, acid fuschin, glacial acetic acid, phosohomolybdic acid and methyl blue .

From the local pharmacy, the following agents were purchased: insulin, normal saline (0.9% NaCl), portable glucometer and glucose test strips (Ascensia Elite^{TM} Bayer AG., Leverkusen, Germany).The Biochemical Research Unit of the University of KwaZulu-Natal, Durban South Africa provided Fluorothane® (Halothane) AstraZeneca Pharmaceuticals, South Africa.

Grapefruit: Ruby grapefruit juice was purchased from a Woolworths Groceries store located in Durban, South Africa. The nutritive value of the grapefruit juice as per the labelling are: contents per 100 ml: Energy 190 Kj; Protein, 0.6 g; Carbohydrate, 10.0 g; Total fat ,0.0 g; Total dietary fibre ,0.4 g; Sodium 0.0 g. The fruit juice contains no preservatives or food additives as per manufacturer's declaration.

Equipment used

- The Nikon Compound Light Microscope attached to a computer
- Freezer (Mallkinckrodt, Ohio, USA)
- Beckman Coulter (Synchron LX20 Clinical Systems, Fullerton, California, USA)
- Makrolon polycarbonate metabolic cages (Techniplats, Labotec, South Africa)
- Novaspec II spectrophotometer (Biochrom Ltd., Cambridge, England)

2.1 Study Design and Procedure

2.1.1 Animal Treatment

Ethical Approval

The experimental procedures and protocol applied in this study were approved by University of KwaZulu-Natal Animal Ethics Research committee and conforms to the "*Guide to the care and used of laboratory animals in research and teaching*". [Published by university of KwaZulu-Natal ethics committee] . Ethics number: 084/11/animal (Ethics approval attached in appendix 1)

2.1.2 Animal Treatment Procedure

56 male *Rattus novergicus* (Wistar rats) that weighed between 250-300 g were obtained and housed at the Biomedical Research Unit (BRU) located at the University of KwaZulu-Natal Westville campus. The animals were divided into two major groups (Diabetic and non-diabetic) with 7 subgroups containing 7 animals per cage. Laboratory conditions of 12 Hour light/dark cycle from 08H00-20H00, temperature 25 ± 2^{0}C and humidity 55±5% were maintained throughout the study period. The animals were allowed free access to commercial rat chow and water *ad libitum*. The different groups were treated with distilled water or regular insulin or grapefruit juice for a period of 8 weeks.

The non-diabetic animals, group 1 (Control) were treated orally with 1.0 ml /Kg BW of distilled water while group 2 (ND-GFJ) were treated orally with 3.0 ml /kg BW of GFJ. The diabetic animals, groups 3, 4, 5 6 and 7 were starved overnight in preparation for the 45 mg/Kg BW or 60 mg/Kg BW of STZ injection. STZ was dissolved in 0.2 ml of 0.1 M Citrate buffer at pH 4.5. Groups 3, 4 and 7 received 60.0 mg/ Kg BW of STZ while groups 5 and 6 received 45mg/kg BW of STZ. Fasting blood glucose concentrations were obtained via tail prick before STZ was administered via a single injection into the peritoneal cavity. Following STZ induction, the diabetic state was confirmed after 3 days by obtaining blood via a tail prick. Animals with fasting blood glucose greater than 6 mmol/L were selected for the study [(39)]. Group 4 (INS- D60) and group 5 (INS- D45) were further treated with 4.0 U/kg BW of insulin by subcutaneous injection (S.C) twice a day while group 3 (D-60) was treated with 1.0 ml /Kg BW of distilled water, Group 6 (GFJ-D45) and group 7 (GFJ-D60) were treated orally with 3.0ml/Kg of BW with GFJ (Table 10).

Group Division	Treatment Groups	Access to water and food	Insulin Treatment	GFJ Treatment	n
Non diabetic male wistar rats	Control	Free access to water and commercial chow	___	___	7
	ND-GFJ	Free access to water and commercial chow	___	3.0 ml/ kg BW b.d	7
STZ-Induced Male Wistar rats	Diabetic control(D-60)	Free access to water and commercial chow	___	___	7
	Ins-D60	Free access to water and commercial chow	4U/Kg BW of insulin b.d SC	___	7
	Ins-D45	Free access to water and commercial chow	4U/Kg BW of insulin b.d sc	___	7
	GFJ-D45	Free access to water and commercial chow	___	3.0 ml/ Kg BW b.d	7
	GFJ-D60	Free access to water and commercial chow	___	3.0 ml/ Kg BW b.d	7

Table 10: Animal Treatment Protocol

On day 57, the rats from each group were housed individually in a Makrolon polycarbonate metabolic cages (Techniplats, Labotec, South Africa) for a period of 24 hours (20H00-08h00). The animals were allowed free access to water *ad libitum* and standard rat chow .The urine was collected for a period of 24 hours in a calibrated reservoir to enable volume determination. The urine samples were stored in a plastic polytops and frozen at -20^{o}C before analysis. Fasting blood glucose (FBG) and glucose tolerance test (GTT) were done on days 1 and 59 respectively in all the treatment groups. Water consumption was monitored on a daily basis and the weights were monitored every two weeks. The study was terminated after 60

days (8weeks) and the final weight for each animal was recorded before the animals were sacrificed by halothane overdose (100 mg/kg). Blood samples were obtained via cardiac puncture while kidney and liver tissues were excised for further analysis. The kidney and the liver were excised from the surrounding connective tissues. The kidney was preserved in formalin and the livers were snap frozen in liquid nitrogen and stored at - 80^{o}C in a freezer (Mallkinckrodt, Ohio, USA) until the day for analysis. This procedure was performed at the biomedical research unit at the University of KwaZulu-Natal in Westville campus.

Chapter 3

3.0. Methods

3.1. Glucose Tolerance Test

The animals were starved overnight and a fasting blood glucose measurement was taken for baseline values. Glucose dissolved in normal saline 3.0g/Kg was administered intraperitoneally in all the treatment groups. The blood glucose levels were monitored by testing the blood obtained via tail prick at times 0, 15, 30, 60 and 90 minutes. The blood was analysed using a portable glucometer and recommended glucose test strips.

3.2. Electrolyte Analysis

The Beckman Coulter machine was first primed before the samples were analysed. Urine samples were thawed at room temperature and a micropipette was used to draw the samples and they were placed in cuvets. Urinary electrolytes (Na^+, K^+, Cl^-), creatinine were analysed using the Beckman Coulter (Synchron LX20 Clinical Systems, Fullerton, California, USA). The estimation of urea involved the hydrolytic degradation of urea in the presence of urease and creatinine determination depended on the reaction of creatinine and sodium picrate to form creatinine picrate. The reagent kits used were purchased from Beckman Coulter, Dublin, Ireland, Inc.

3.3. Liver Glycogen Determination

Hepatic glycogen concentration method was adopted and modified from Ong and Khoo (2000) methodology [248]. Liver tissue samples weighing between 0.5-1.0 g were placed in a pyrex test tube. A volume of 1.5 ml of 30% potassium hydroxide saturated in sodium sulphate was added into the test tube to enable homogenization and boiled in a water bath (100^0C) for 20 minutes. The samples were then cooled inside an ice box. Then 2.0 ml of 95% ethanol was added to each sample to deproteinize it. The sample was then left to stand on ice for approximately 30 minutes. The mixture was then centrifuged for 20 minutes at 840 rpm. The supernatant was aspirated and the white precipitate (glycogen) that remained in the tube, 1000 µL of water was added to it. A volume of 100 µl of dissolved glycogen was then taken in triplicate and added onto the test tube then 900 µL of water was added. A volume of 1.0 ml

of 5% phenol was added to all test tubes. Then 5 ml of 98% sulphuric acid was added after 30 seconds and left to stand for 10 minutes then shaken at room temperature for a further 10 minutes. The mixture was then added onto a cuvet and placed into a Novaspec II spectrophotometer (Biochrom Ltd., Cambridge, England) and a reading was taken at 490nm. A standard glycogen stock solution was prepared using glycogen powder and it received the same treatment as the tissue samples. From this a standard curve was drawn at it was used to determine the glycogen concentrations (1mg/ml-1µg/ml) in samples. Blank samples were made by replacing the sample with 2 µL of distilled water.

3.4. Plasma Insulin

Blood collected from heparin containing vacutainer was centrifuged at room temperature for 20 minutes at 15000rpm. The method of plasma insulin analysis was based on a direct sandwich with two monoclonal antibodies that are directed against separate antigenic determinants on the insulin molecules. The insulin molecule reacts with peroxidase-conjugated anti-insulin antibodies that bind to microtitre wells on incubation. The enzymes that were unbound were removed by washing while the bound conjugates were detected by reaction with 3, 3', 5, 5'-tetramethylbenzidine. 0.5 M Sulphuric acid was added to stop the reaction and the resultant coloured product was analysed spectrophotometrically at a wavelength of 450nm.

3.5. Tissues Preparation and Staining

The renal tissue was cut sagittally using a microtome with each section 2µm thick. The sections were then mounted onto a microscope slide after being unfolded in a warm water bath. The microscopes slides were then heated to ensure proper adhesion of the tissue onto the slide. The microscopic slides were stained using Hemotoxylin and Eosin (H&E) and Masson's Trichrome Stain (MTS). Each slide was stained using both stains. The reagents and protocol for the staining procedures are outlined in appendix 2.

3.5.1 Image Analysis

The stained slides were viewed using The Nikon Compound Light Microscope at the University of KwaZulu-Natal's Electron Microscope Unit (EMU) equipped with a camera for image capture. The images were then analyzed by a pathologist.

3.6. Calculations

To determine the Glomerular filtration rate (GFR) the urinary clearance of creatinine and plasma creatinine values were used to determine the filtration rate using the formula below.

$$\text{Creatinine clearance (ml/min)} = \frac{\text{urinary Creatinine (mmol/L)} \times \text{urine volume (ml)}}{\text{Plasma Creatinine (mmol/L)}}$$

Area under the curve (AUC) = Calculated from glucose–time curves presented as AUC units (mM X minutes)

3.7. Statistical Analysis

The baseline values were obtained from untreated non-diabetic and STZ-Induced diabetic rats. Data obtained will be presented as mean ±SD. Statistical analysis was done by One-way ANOVA or non-parametric Mann Whitney V, Student's *t*-test, where applicable, using Graphpad Prism®, V5.0 (Graphpad Prism® Software, Inc. San Diego CA). A probability level of $P < 0.05$ was considered to be statistically significant.

Chapter 4

4.0 Results

4.1 Animal Growth and Water Consumption

After the induction of diabetes the weights of the animals changed significantly with diabetic animals (D-60 &D-45) exhibiting significant (*P<0.0001) loss in body mass when compared to the control. Treatment with GFJ significantly ($^{\Omega}$P=0.0020) improved the body mass in diabetic group GFJ-D60 when compared to group D-60. In the type 2 diabetic models (GFJ-D45 vs D45) treatment with GFJ improved the weight but the difference was not statistically significant. GFJ treatment did not affect the growth of non-diabetic animals (GFJ-ND) when compared to the control (Figure 19). Similarly treatment with insulin (INS-D60) significantly (^P <0.0001) improved weight of the diabetic animals when compared to non-treated diabetic animals group (D-60).

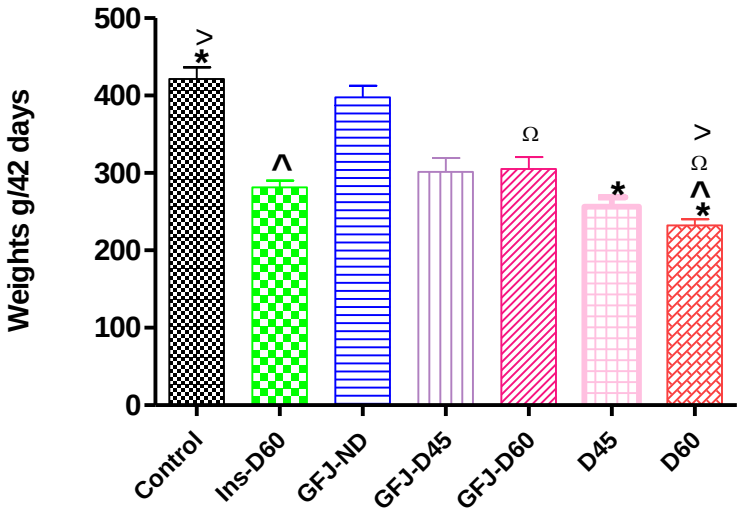


Figure19: Animal growth at the end of 8 weeks of treatment. INS-60, insulin- diabetic 60mg STZ; GFJ- ND, Grapefruit juice non-diabetic; GFJ-D45, Grapefruit juice -diabetic 45mg STZ ; GFJ-D60, Grapefruit juice diabetic 60mg STZ; D-45 , diabetic – 45mg STZ; D-60 , Diabetic -60mg STZ. * P<0.0001; ^ P=0.0072; # P=0.0036; $^{>}$P<0.0001;$^{\Omega}$P=0.0020

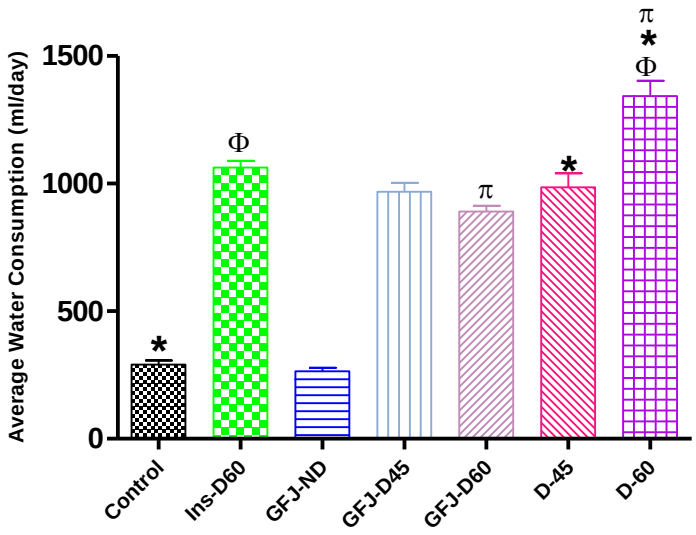


Figure 20: Average daily water consumption in treatment groups. INS-60, insulin- diabetic 60mg STZ; GFJ- ND, Grapefruit juice non-diabetic; GFJ-D45, Grapefruit juice -diabetic 45mg STZ ; GFJ-D60, Grapefruit juice diabetic 60mg STZ; D-45 , diabetic – 45mg STZ; D-60 , Diabetic -60mg STZ. $^{\pi*,\Phi,\wedge}P<0.0001$

The diabetic animals (D-45 and D-60) exhibited significantly ($^{*}P<0.0001$) increased water consumption when compared to the control (Figure 20). Treatment of the diabetic animals (GFJ-D60) with GFJ reduced the water consumption significantly ($^{\pi}P<0.0001$) compared to the non-treated diabetic group (D-60). Water consumption in the non-diabetic animals treated with GFJ was similar to the control while treatment with insulin significantly ($^{\Phi}P<0.0001$) reduced the consumption of water.

4.2 Fasting Blood Glucose and Fasting Plasma Insulin

Fasting blood glucose (FBG) was significantly increased (^ P<0.0001) in the diabetic animal groups (D-60) and D-45 (*P=0.0002) when compared to the control respectively. Treatment of the diabetic animals with GFJ (GFJ-D60 vs D-60) and (GFJ-D45 vs D45) lowered FBG significantly $^{>}P<0.0001$, $^{\#}P=0.0329$ respectively when compared to their respective controls (Figure 21). FBG concentration in non-diabetic rats treated with GFJ (GFJ-ND) was similar when compared to the control. On the other hand fasting plasma insulin was significantly lower in the diabetic group (D-60) (^P<0.0001) and group (D-45) (*P=0.0002) when compared to the controls. Treatment with GFJ did not improve fasting plasma insulin in the

diabetic groups compared to the non-treatment diabetic group (D-45 and D-60). Insulin treatment on the other hand improved FPI significantly (*P<0.0001) when compared to the control (D60) Figure 22.

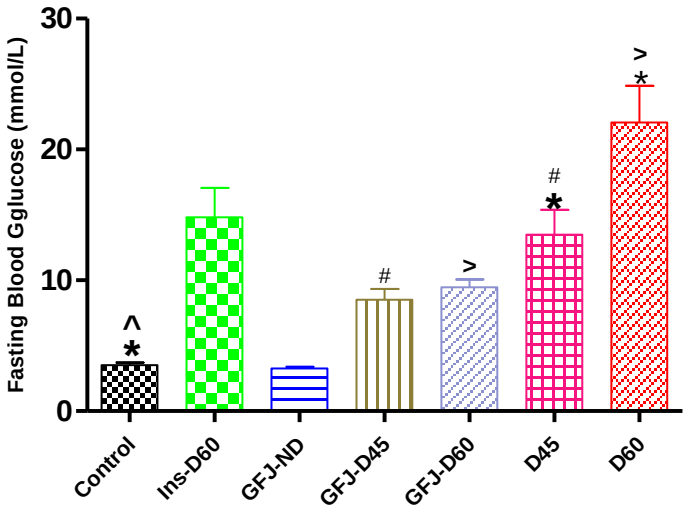


Figure 21: The fasting Blood glucose levelsINS-60, insulin- diabetic 60mg STZ; GFJ- ND, Grapefruit juice non-diabetic; GFJ-D45, Grapefruit juice -diabetic 45mg STZ ; GFJ-D60, Grapefruit juice diabetic 60mg STZ; D-45 , diabetic – 45mg STZ; D-60 , Diabetic -60mg STZ.*p<0.0001; # p= 0.045; ^ p =0.007

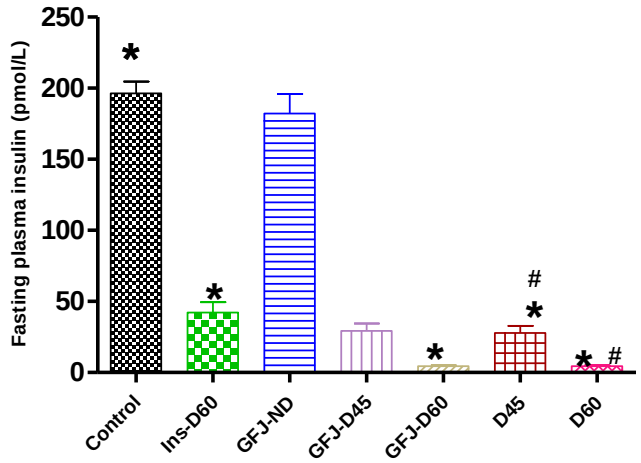


Figure 22: Fasting plasma insulin levels. INS-60, insulin- diabetic 60mg STZ; GFJ- ND, Grapefruit juice non-diabetic; GFJ-D45, Grapefruit juice -diabetic 45mg STZ ; GFJ-D60, Grapefruit juice diabetic 60mg STZ; D-45 , diabetic – 45mg STZ; D-60 , Diabetic -60mg STZ. *P<0.0001; # p =0.0009

4.3 Glucose Tolerance Test

Calculated area under the curve (AUC) from GTT curves show that diabetic animals (D-45 and D-60) experienced significant (*P<0.0001) glucose intolerance in comparison to the control group (Figure 23 A and B). Treatment with GFJ significantly (*P<0.0001) improved glucose intolerance in diabetic groups (GFJ-D 60) and (GFJ-D45) with GFJ when compared to the non-treated diabetic group (D60 and D-45) respectively (Figure 23).

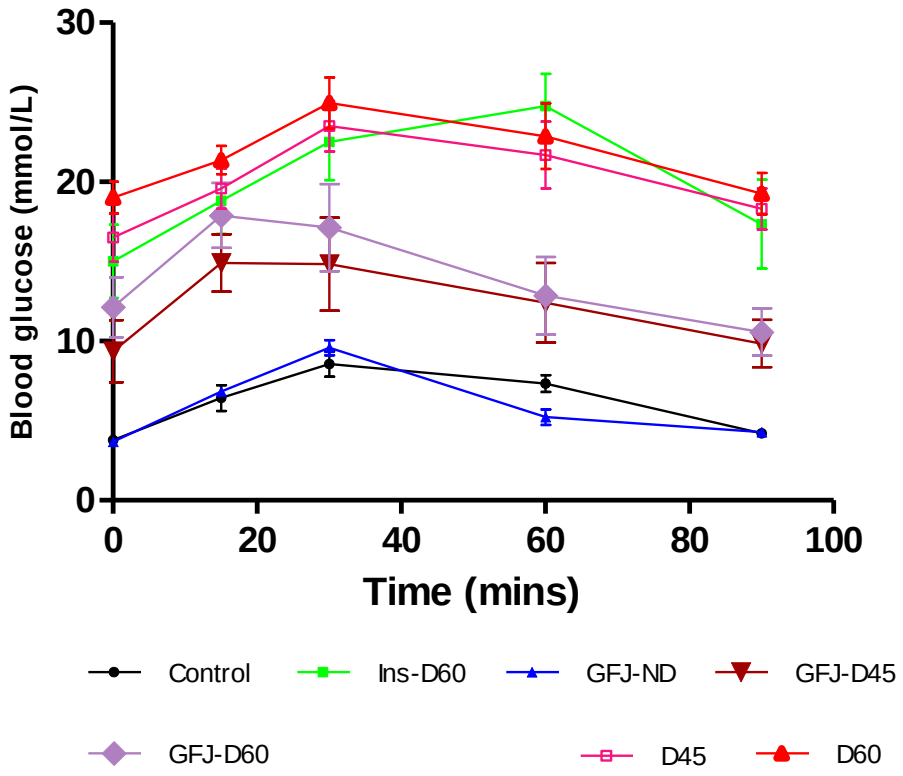


Figure 23 A: Glucose Tolerance Test.INS-60, insulin- diabetic 60mg STZ; GFJ- ND, Grapefruit juice non-diabetic; GFJ-D45, Grapefruit juice -diabetic 45mg STZ ; GFJ-D60, Grapefruit juice diabetic 60mg STZ; D-45 , diabetic – 45mg STZ; D-60 , Diabetic -60mg STZ.

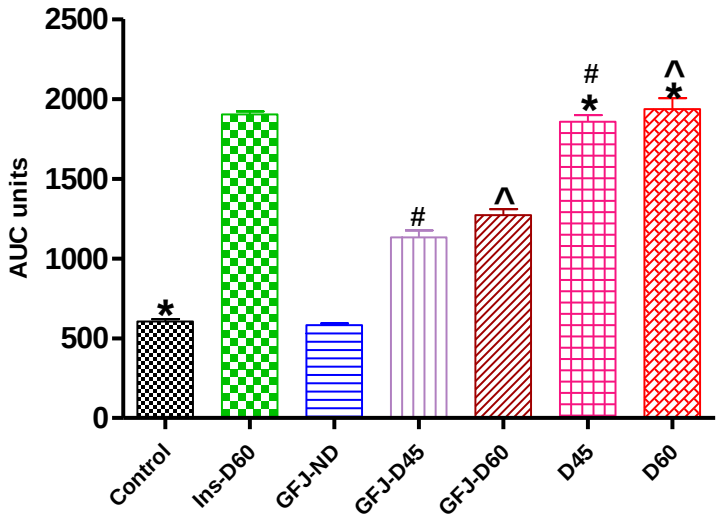


Figure 23 B: Area under the curve (AUC) calculated from the fasting blood glucose levels. INS-60, insulin- diabetic 60mg STZ; GFJ- ND, Grapefruit juice non-diabetic; GFJ-D45, Grapefruit juice -diabetic 45mg STZ ; GFJ-D60, Grapefruit juice diabetic 60mg STZ; D-45 , diabetic – 45mg STZ; D-60 , Diabetic -60mg STZ.(*P<0.0001; # p=0.0012; ^ p=0.0034)

4.4 Glycogen Concentration in Hepatic Tissue

Treatment	**Hepatic Glycogen Concentration(µg/ml)**		**n**
Non-diabetic male wistar rats	Control	4.2±0.3*^	7
	ND-GFJ	5.7±0.2*^	7
STZ-Induced male Wistar rats	Diabetic control(D-60)	3.2 ± 0.5*	7
	Ins-D60	3.6 ± 07*	7
	GFJ-D60	4.9 ±0.5*	7

*P value <0.05 vs Control, ^P value=0.0013

Table 11: Effects of 8 week treatment with GFJ and insulin on hepatic glycogen concentration in non-diabetic and STZ- induced Diabetic rats.

The hepatic glycogen content in the diabetic control (D-60) was significantly (P=0.024) lower than the control group Table 11. Treatment with GFJ significantly (P=0.00016) increased hepatic glycogen content in the diabetic group (GFJ-D60) when compared to the untreated group (D60). In the non-diabetic animals (GFJ-ND), GFJ significantly increased (P=0.0013) hepatic glycogen content when compared to the control.

4.5. Urinary Electrolytes

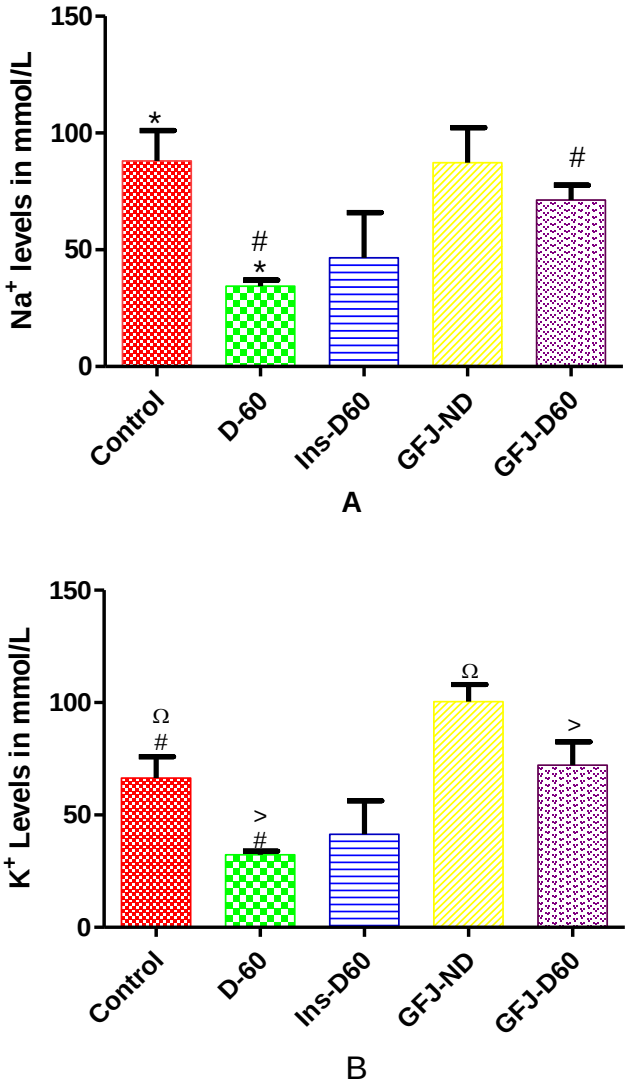
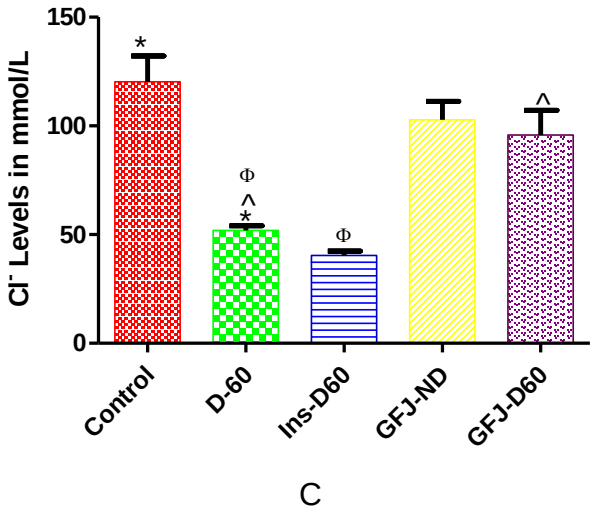


Figure 24: Urinary sodium (Na^+) and potassium (K^+) levels. INS-60, insulin- diabetic 60mg STZ; GFJ- ND, Grapefruit juice non-diabetic; GFJ-D60, Grapefruit juice diabetic 60mg STZ; D-60 , Diabetic -60mg STZ

Urinary excretion of sodium (Na^+), potassium (K^+) and chloride (Cl^-) were significantly decreased (*P = 0.032, $^{\#}$P= 0.0075 and *P=0.0003 respectively) in diabetic group (D-60) in

comparison to the control group. GFJ treatment of group (GFJ-D60) caused a significant ($^{\#}$P= 0.0003, $^{>}$P=0.0049 and ^P=0.0046) increase in the excretion of Na^+ , K^+ and Cl^- respectively when compared to the untreated diabetic group D60 (Figure 24 and Figure 25 A). In the non-diabetic group (GFJ-ND), GFJ significantly increased ($^{\Omega}$P=0.0165) urinary K^+ but not Na^+ and Cl^- when compared to the control. Treatment with insulin did not cause a change in the urinary levels of Na^+ and K^+ but significantly ($^{\Phi}$P=0.0036) increased urinary Cl^- in diabetic group INS-D60 when compared to D60.



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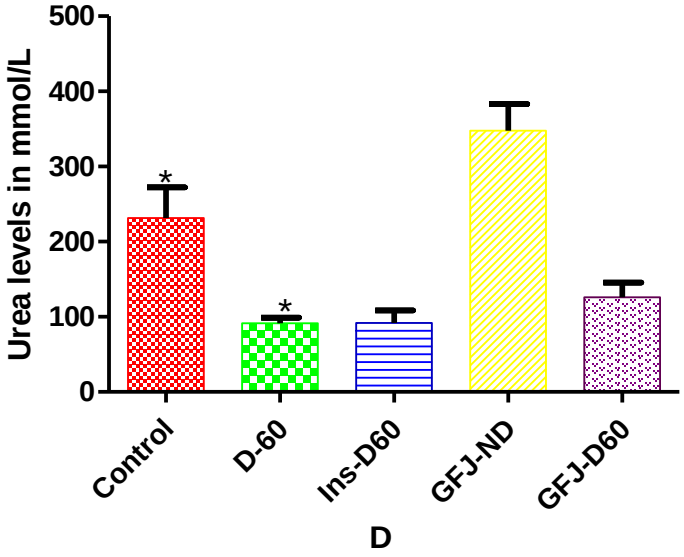
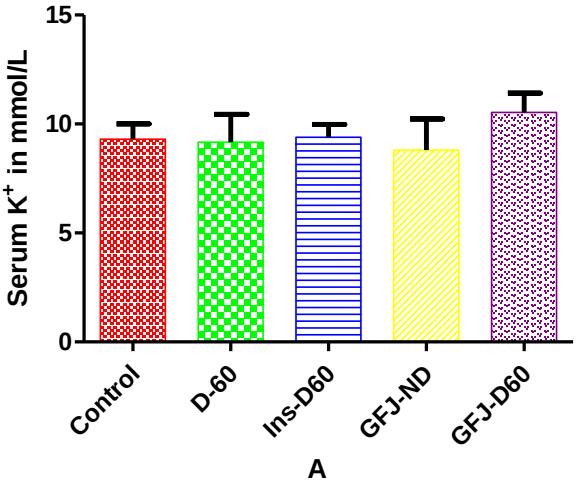


D

Figure 25: Urinary chloride (Cl^-) and urea levels. INS-60, insulin- diabetic 60mg STZ; GFJ-ND, Grapefruit juice non-diabetic; GFJ-D60, Grapefruit juice diabetic 60mg STZ; D-60 , Diabetic -60mg STZ. p*<0.0001,P^=0.0023

Urinary urea content was significantly (*P=0.0073) reduced in the diabetic group (D60) when compared to the control group. Treatment with GFJ (GFJ-D60) or insulin (INS-D60) did not cause a change in urinary urea excretion compared to group (D-60).

4.6. Serum Electrolytes



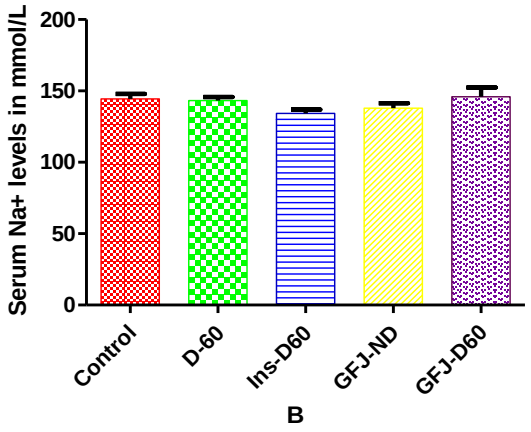


Figure 26: Serum Potassium K^+ and Sodium (Na^+) levels.INS-60, insulin- diabetic 60mg STZ; GFJ- ND, Grapefruit juice non-diabetic; GFJ-D45, Grapefruit juice -diabetic 45mg STZ ; GFJ-D60, Grapefruit juice diabetic 60mg STZ; D-45 , diabetic – 45mg STZ; D-60 , Diabetic -60mg STZ

The serum electrolytes concentration of Na^+ and K^+ at the end of 8 week treatment with GFJ or insulin was not affected in all the groups (Figure 26).

4.6.1 Creatinine Levels

Treatment	Glomerular Filtration rate (GFR) ml/min		Urinary creatinine (mmol/L)	Serum creatinine (mmol/L)	n
Non diabetic male wistar rats	Control	1.9±0.2	6.32±0.5	39.17±2.6*	7
	ND-GFJ	1.8±0.6	7.3±1.2	32.00±1.3*	7
STZ-Induced Male Wistar rats	Diabetic control(D-60)	Low urinary creat	Low	33.80±1.5	7
	Ins-D60	Low urinary creat	Low	36.17±5.1	7
	GFJ-D60	1.59±0.03	4.37±0.8	33.50±3.5	7

Table 12: Effect of Grapefruit Juice on Urinary Creatinine and Glomerular Filtration Rate.

Urinary creatinine in the STZ-induced diabetic animals was too low and could not be detected by the analytical method used. Treatment of the diabetic groups with insulin did not alter urinary or serum creatinine but treatment with GFJ significantly (P=0.0266) increased serum and urinary Creatinine in the diabetic group (GFJ-D60) when compared to D60.

4.7 Histological Sections of the Kidney

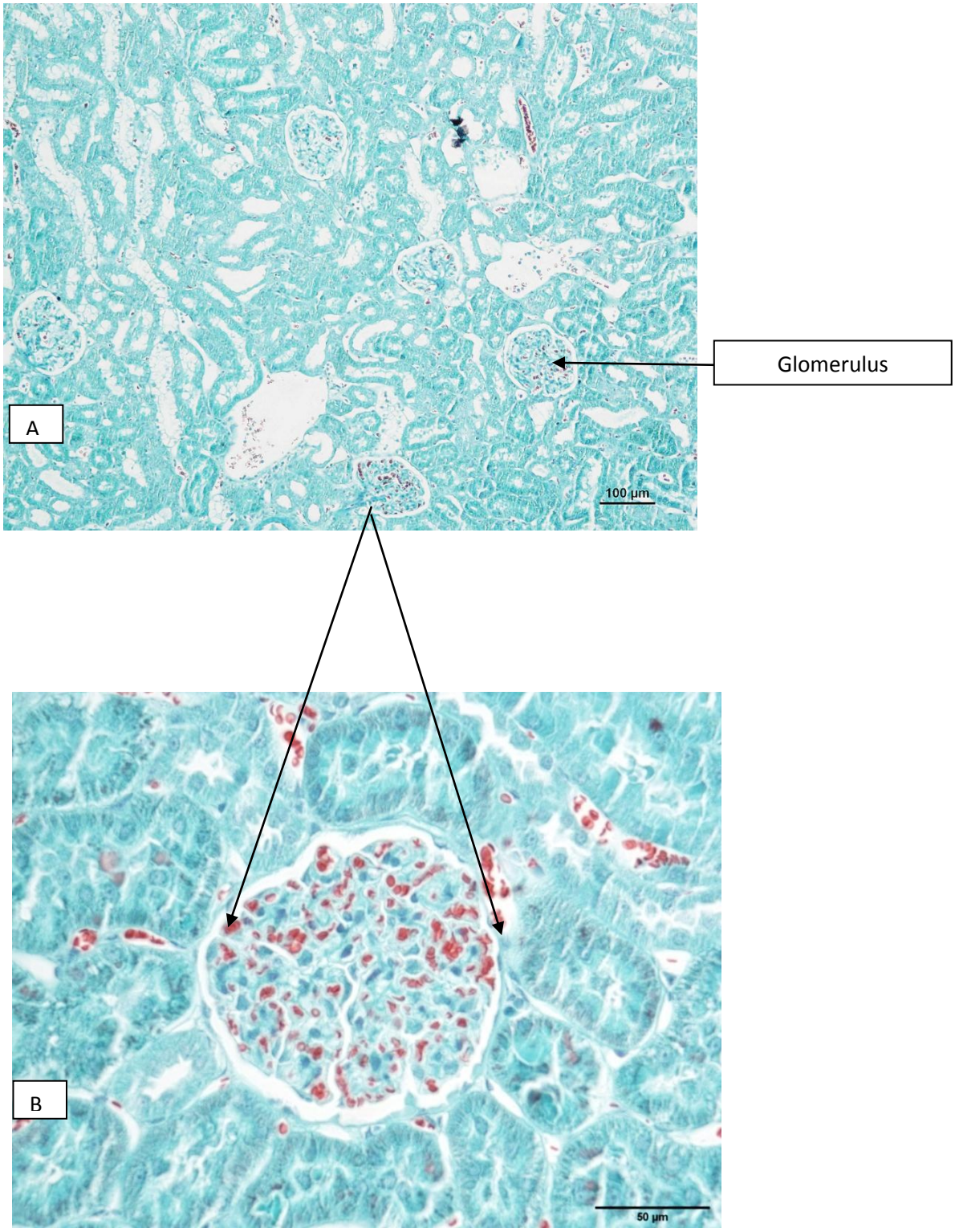


Figure 27: Photomicrograph of renal tissue of normal glomerular morphology control group low magnification (A) and high magnification X40 (B)

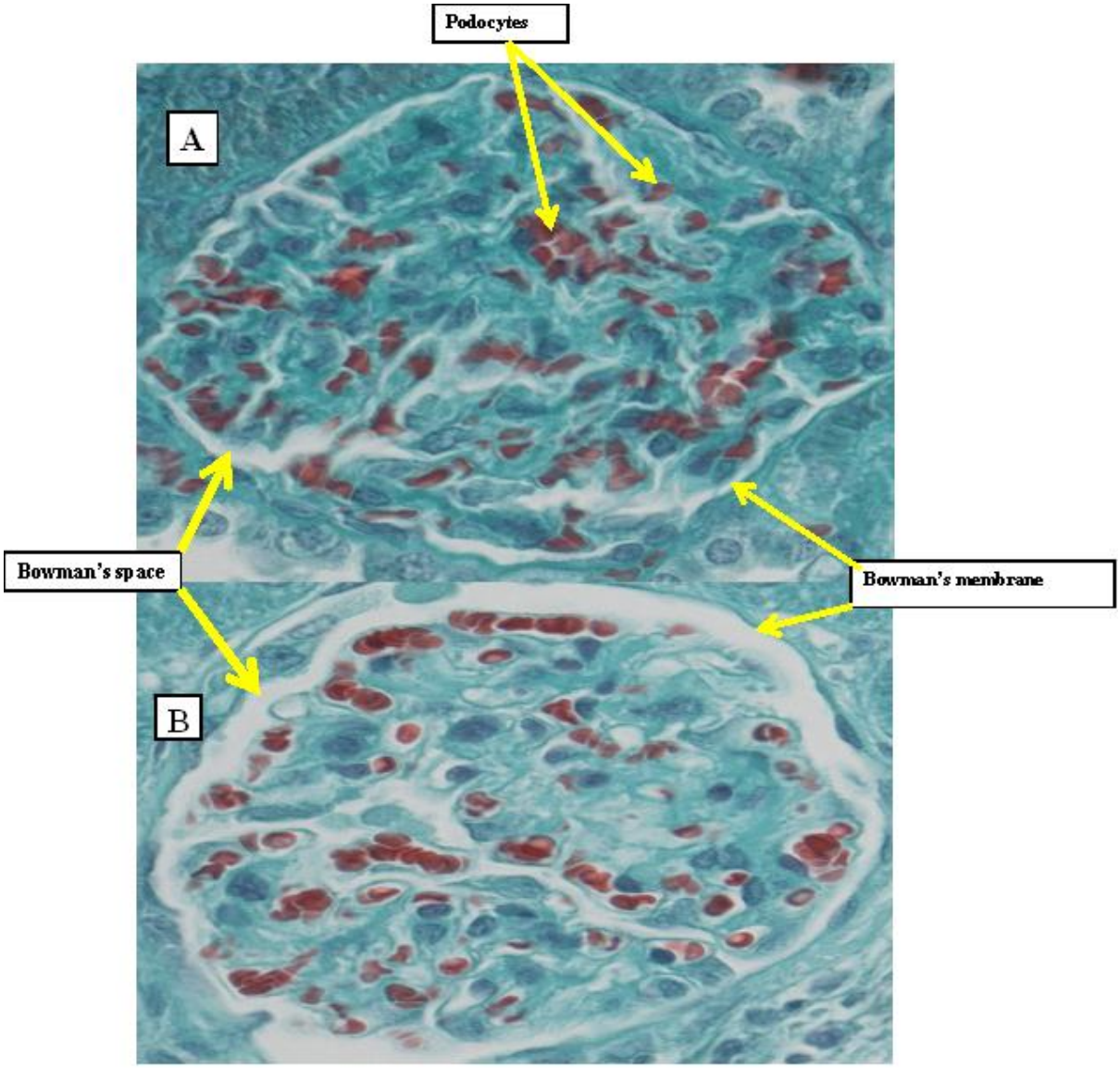


Figure 28: Renal histologic lesions showing glomeruli hypertrophy in diabetic rats that were not treated (A) and normal glomeruli in those that were treated with GFJ (B).

Part A of Figure 28 shows the morphological changes that took place in the untreated diabetic animal group (D-60). Treatment of the diabetic animals with GFJ –D60 prevented the development of renal hyper trophy (Figure 28 B). The structural morphology of the glomeruli in the he control group (Figure 27) were similar to GFJ-treated group.

Chapter 5

5.0 Discussion

5.1. General

The aim of this study was to investigate the effects of grapefruit juice on hyperglycaemia and renal water and electrolyte handling in STZ-induced diabetic rats. Results from this study show that grapefruit juice lowered the blood glucose levels and consequently improved glucose intolerance in type1 and 2 diabetic models (GFJ-D45 and GFJ-D60) while improving renal electrolyte handling in STZ-induced diabetic rats.

5.2. Hypoglycaemic Effects

The administration of streptozotocin (STZ) 60 mg/Kg BW and 45 mg/Kg BW was done to produce type 1 (D60) and type 2 (D45) diabetic models respectively in this study. The diabetic animals showed initial decrease in body weight but the type 1(D60) had significantly lost weight (Figure19) when compared to the control group and this could be due decreased effects of insulin action. Plasma insulin levels were measured in all the treatment groups with the diabetic groups (D45 and D60) having the lowest plasma insulin concentration (Figure 22) inferring β-cell loss. Steptozotocin's affinity and specific toxicity to pancreatic β-cells resulted in the complete or partial destruction of the pancreatic β-cells resulting in decreased insulin production. Decreased insulin levels in the body are associated with an increase in plasma blood glucose concentration, decreased storage and synthesis of carbohydrates, proteins and lipids [(138)]. The difference in body weight between type1 and type 2 diabetic models was due to the presence of low levels of insulin and or residual insulin in the D-45 group resulting in decreased lipolysis and proteolysis processes in the tissues (Figures 19 & 22). Weight reduction in diabetic group (D60) was possibly due to enhanced proteolysis, lipolysis associated with insulin deficiency. The treatment with GFJ or insulin resulted in improved weight gain. This suggests that GFJ could possibly reduce proteolysis and or lipolysis but the mechanism of action is still unclear. Fasting plasma insulin levels did not increase with the administration of GFJ suggesting that GFJ is neither insulinotropic nor β-cell regenerative. In this study animal treatment with GFJ or insulin improved the weights of the diabetic animals and treatment of non-diabetic animals did not cause a significant change in the body weight compared to the control suggesting that GFJ does not affect the normal growth of the animals (Figure 19).

Excessive urination, was accompanied by polydipsia in the diabetic animals suggesting the presence of a diabetic state. These changes could particularly be attributed to the counter-regulatory mechanisms in which the body tries to correct dehydration [26]. GFJ significantly reduced polydipsia in the diabetic group (D60-GFJ) compared to the non-treated diabetic group (D-60). Decreased polydipsia suggests improved blood glucose concentration in the diabetic animals due to improved diuresis. A decrease in plasma insulin levels was accompanied by an increase in FBG and glucose intolerance in diabetic group (D-60) when compared to the control group (Figures 21 and 23 B). Treatment with GFJ lowered FBG and improved glucose intolerance in diabetic groups (GFJ-D60 and GFJ-D45) when compared to untreated diabetic groups (D60 and D45) respectively. This was effect was not apparent in the insulin treatment group (INS-D60), where only FBG was reduced but glucose intolerance was still evident when compared to D60 (Figure 21 and 23B). This observation could be due to the fact that the dose of exogenous insulin (4U/Kg BW S.C b.d) used was not sufficient to improve glucose intolerance. Fasting plasma insulin was not affected when the non –diabetic group (GFJ-ND) was treated with GFJ compared to the control and when GFJ-D60 was treated and compared to group D-60. This suggests that GFJ does not stimulate insulin release and its hypoglycaemic effects could be due to other mechanisms like activation of AMPK. Previously the hypoglycaemic effect of GFJ was observed and reported by Owira and Ojewole 2009 (37) from our laboratory when they compared GFJ effects and metformin in non-diabetic rats. It was also observed that GFJ did not increase plasma insulin levels in non–diabetic rats. The current results confirm similar trend in diabetics.

The hepatic glycogen content in the diabetic group D60 was lower when compared to the control. However, GFJ treatment increased hepatic glycogen concentration in both diabetic (GFJ-D-60) and non-diabetic (GFJ-ND) when compared to groups D-60 and control respectively (Table 11). The mechanism of action by which GFJ lowers blood glucose is not currently known but the results suggests that GFJ increases hepatic glycogen formation in hepatic tissues of diabetic and non-diabetic rats. This could be due to grapefruits effects on the key glycolytic enzymes involved in the metabolism of glucose in the liver. Studies have shown that flavonoids like naringenin and hesperitin found in grapefruit have the ability to lower blood glucose levels (30) (50) (79). Naringenin has been known to decrease the hepatic activity of glucose-6-phosphatase (61). In the liver glucose homeostasis depends upon the rate of gluconeogenesis and glyconeolysis (249). In the diabetic state enzymes like hexokinase,

glucokinase and phosphofructokinase don't function to their full potential because of insufficient insulin in the body [250] causing a decrease in hepatic glycogen concentration [251]. In this study we did not determine to mechanism of action in which GFJ increases glycogen content and further studies will be required to elucidate grapefruits juice exact mechanism(s) of action.

5.3. Effects of Grapefruit Juice on Renal Function

Chronic hyperglycaemia was accompanied by a decrease in urinary electrolytes (Na^+, K^+ and Cl^-) and GFR in untreated diabetic group (D60) when compared to the control group (Figure 24 and 25 B). Treatment of the diabetic group GFJ-D60 with GFJ increased urinary Na^+, K^+ and Cl^- when compared to the untreated diabetic group (D60) but in the non-diabetic group (GFJ-ND) GFJ treatment caused an increase in urinary K^+ but not Na^+ and Cl^- when compared to the control group. On the other hand insulin treatment of group (Ins-D60) caused an increase in urinary Cl^- but not Na^+ or K^+ when compared to the untreated diabetic group D60. Increased urinary output of K^+ was also observed in the non-diabetic rats and this effect is common with the consumption of fruit juices [80]. To evaluate renal function urinary and serum creatinine in the diabetic group (D60) was analysed and urinary creatinine was undetectable by the analysis method used. In addition treatment with insulin did not change urinary creatinine but it increased serum creatinine levels. GFJ treatment on the other hand increased urinary creatinine but not serum creatinine . These results suggest that the STZ-induced diabetic rats developed diabetic nephropathy in the course of the 8 week study period. Sodium retention, chronic hyperglycaemia and reduced GFR are signs of renal dysfunction observed in animal models with declining renal function [252] .

GFJ treatment in group GFJ-D60 caused an increase in urinary Na^+ which was accompanied by an improvement in the GFR when compared to the untreated group D60. However insulin treatment did not improve GFR or urinary Na^+ concentration. This could be explained by the fact that insulin possesses antinatriuresis properties independent of the actions aldosterone and GFR [231]. Consequently serum electrolytes in the treated and untreated diabetic groups were not affected. This observation could be due to the fact that nephropathy was not severe enough to cause changes in the serum electrolyte levels or the body's counter regulatory mechanism was enough to correct the electrolyte imbalance. Na^+ plays a major role in the determination of blood osmolality because it is the principal osmole in the extra cellular fluid (ECF) and it is coupled together with glucose during transportation in the renal tubules.

Increased Na^+ reabsorbed in the proximal convoluted tubule is observed in the early stages of DM resulting in reduce urinary output due to tubular hypertrophy caused by increased glucose concentrations [253] [254]. The inhibition of Na^+ transporters in the proximal tubule in conjunction with reduced hyperglycaemia could be a major cause of decreased Na^+ reabsorption and increased urinary excretion. Sodium and glucose are transported together in the renal tubules via the SLGT1 and SLGT2 [255] [252] and a study done by Li *et al.* [80] showed that the flavonioid naringenin abundantly found in GFJ inhibited renal and intestinal SLGT [80]. Renal tissues exposed to high glucose concentrations for a prolonged period of time are prone to structural and physiological changes. Severe hyperglycaemia observed in the diabetic animals is accompanied by changes in the brush border membrane to cater for the increased glucose load due to the up-regulation of SGLT2 in the proximal tubule brush border [80].

Glucose efflux from the tubule relies on the gradient created by Na^+ dependent influx due to outwardly directed glucose gradient. Glucose induced toxicity can cause tubular injury (proximal tubules) in the kidneys of diabetic patients [255]. The proximal tubule is the first segment of the nephron and helps in the maintenance of extracellular fluid volume. Animal studies done have shown that prolonged hyperglycaemia leads to the disruption of the tubule-glomerular feedback mechanism in the renal tissues [253]. Tubuloglomerular feedback involves the changes in the SNGFR due to change in the concentration of Na^+, Cl^- and K^+ in the glomerular filtrate. Change in the tubular electrolyte concentration of Na^+, K^+ and Cl^- is caused by fractional reabsorption in the atrophic proximal tubules and this has been observed in IDDM and STZ-induced wistar rats [251]. The change in fluid electrolyte concentration is sensed by the macula densa cells located in the distal convoluted tubule [256] and this causes an increase in single nephron glomerular filtration rate [252] [257]. Water and sodium re-absorption is increased with an increase in glucose concentration and this can be observed in diabetics [225]. A reduction in Sodium reabsorption causes a decrease in the glomerular filtration rate (GFR) due to the disruption of the normal renal perfusion processes [252]. Metabolic waste products like urea and creatinine are excreted principally by the kidney and renal injury is associated with reduced excretion and a concurrent reduction in GFR [258]. Creatinine is a product of non-enzymatic dephosphorylative cyclization of phosphocreatine and urea levels are increased in the plasma are as a result of increased proteolysis accompanied by impaired nitrogen balance in diabetic patients due to reduced anabolic

actions of insulin [207]. Creatinine has been used over the years to calculate creatinine clearance to evaluate kidney functions and disease conditions like glomerulonephritis, tubular necrosis and dehydration have been associated with low creatinine values [259]. Low Glomerular filtration rate in patients with renal problems has been accompanied by fluid and electrolyte imbalances observed in the diabetic animals [258] [260].

The transport of electrolytes in the kidney is mediated via different transport system in combination with neurohomanal influences. Renin and antidiuretic hormones (vasopressin, aldosterone) play an important role in the hormonal regulation of fluid electrolytes and water balance [261]. Basolateral mechanism involving Na^+-K^+-ATPase establishes Na^+ gradient for transcellular Na^+-Cl^- transport by the proximal tubule and an exit for Na^+ and the diabetic state has been shown to increase its activity in the kidney [253]. In diabetic nephropathy activation of the PKC pathway, Na+/H exchanger activity, alteration of cell alkalinisation and change in intracellular calcium contribute to renal tubular hypertrophy and a consequent reduction in kidney functions [254].

The changes in the renal electrolytes were accompanied by structural changes in the glomerulus as seen in figure 28 A of the untreated diabetic animals (D-60). Treatment with GFJ prevented the formation of histologic lesions GBM thickening and glomerular hypertrophy Figure 28 B. The results of this study provide the first *in vivo* evidence that suggests GFJ can ameliorate diabetic nephropathy by improving Na+ handling, GFR and chronic hyperglycaemia in STZ-induced diabetic rats.

Chapter 6

6.0. Conclusion

The results obtained in this study showed grapefruits versatility as an antidiabetogenic agent. It managed to lower hyperglycaemia while improving glucose intolerance in STZ-Induced diabetic animals. Renal dysfunction observed in the diabetic animals was improved with GFJ treatment; Na+ retention was reversed accompanied by increased GFR as seen in the treatment groups. GFJ prevented the formation of histological lesions that contribute to the progression and development of diabetic nephropathy. The mechanism of actions in which GFJ works can be speculated to increase hepatic glycogen content and it might inhibit SGLT2 in the proximal convoluted tubule and prevent oxidative damage due to its powerful antioxidant effects ameliorating renal hypertrophy.

6.1. Limitations of the Study

The study did not investigate the mechanism of action in which GFJ lowers the blood glucose concentration and its effects on the glycolytic enzymes glucose-6-phosphatase and hepatic glucokinase. Lithium clearance was not performed on the STZ-Induced diabetic rats to determine the fractional excretion of sodium from the tubules as a measure of renal function. This was because .of time constraints and limited funds.

6.2. Future Prospects and Recommendations for Further Study.

This study has contributed to the body of knowledge in terms of the pharmacological effects of grapefruit juice in diabetic nephropathy but some questions are yet to be answered on the effects of *C. paradisi* on glycolytic enzymes; glycogen synthase and hepatic glucokinase. Further studies should be conducted on the effects of GFJ on proximal tubular Na^+ handling and renal hormones that affect Na^+ homeostasis (Aldosterone and vasopressin).Determination of Renal Glomeruli PKC Activity.

Chapter 7

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Chapter 8

Appendix 1 – Ethical Approval

UNIVERSITY OF
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YAKWAZULU-NATALI

Research office
Animal Ethics Research Committee

Govan Mbeki Centre, Westville Campus,
University Road, Chiltern Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2273/35 Fax (031) 260-2384
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15th June 2011

Reference: 084/11/Animal

Dr. P. Owira
Department of Pharmacology
School of Pharmacy and Pharmacology
Block F2 521
Westville Campus
UKZN

Dear Dr P. Owira

Renewal: Ethical Approval of Research Project on Animals

I have pleasure in informing you that on recommendation of the review panel, the Animal Ethics Sub-committee of the University Research and Ethics Committee has granted ethical approval for 2011 on the following project:

1. Potential Modulation of the expression organic cation transporter protein(OCT1), P-Glycoprotein, and Cytochrome P450 (CYP3A4) in the rat liver by grapefruit juice *in vivo*.
2. Effects of grapefruit juice on serum lipid profile, Glycemic index and metformin-induced ;actic acidosis in non-diabetic rats.

Yours sincerely

Prof. Theresa HT Coetzer (Chair)
ANIMAL RESEARCH ETHICS COMMITTEE

Cc Registrar, Prof. J Meyerowitz
Research Office, Mr Nelson Moodley
Head of School, Prof. F. Sulleman
Biomedical Research Unit, Dr. S. Singh

Appendix 2- Author Permissions

Richardson, Amy [RichardsonA4@uthscsa.edu] on behalf of DeFronzo, Ralph A [defronzo@uthscsa.edu]

06 February 2013 06:46 PM

Julia,

Permission granted.

Sincerely,

Ralph A DeFronzo, M.D.

Amy Richardson

Assistant to Ralph A DeFronzo, M.D.

Phone: 210-567-6691 | Fax: 210-567-6554

richardsona4@uthscsa.edu

Dear Dr Defronzo,

My name is Julia Hayangah from the University of KwaZulu-Natal in Durban, South Africa. I would
like to ask for your permission to use one of your illustrations published in the journal of Diabetes Obesity and Metabolism 14:5-14, 2012 for my master's thesis. I would like to use figure 2 that depicts renal glucose absorption.

Journal title:
Defronzo, R.A., Davidson , J.A. and Del Prato, S.: The Role of The Kidneys in Glucose Homeostasis: A New Path Towards Normalizing Glycaemia. *Diabetes ,Obesity and Metabolism.14:5-14,2012.*

Yours Sincerely

Julia Hayangah

Jones, Jennifer (ELS-OXF) [J.Jones@elsevier.com]

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Bailey, Clifford J [c.j.bailey@aston.ac.uk]

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Michael Brownlee [Michael.Brownlee@einstein.yu.edu]

TO : Julia Hayangah

24 October 2012 07:31 PM

Dear Julia,

Thank you for your interest. Permission granted.

Best regards

Julia Hayangah

Actions
To: brownlee@aecom.yu.edu
Cc:Peter Owira
Sent Items
24 October 2012 06:36 PM

Dear Dr Brownlee,

My name is Julia Hayangah from the university of Kwazulu-Natal in Durban South Africa. I would

like to ask for your permission to use one of your illustrations published in Nature vol 414 December 2001 for my master's thesis. i would like to reuse figure 1 on pg 814 that depicts the Aldose reductase and the polyol pathway.

Journal article

" Brownlee , M.: Biochemistry and molecular cell biology of diabetic complications. Nature 414: 813-820 . December 2001.

Your assistance will be highly appreciated .

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so granted.

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On Oct 8, 2012, at 8:36 AM, Julia Hayangah wrote:

Dear Saltiel and Khan,

My name is Julia Hayangah from the university of Kwazulu-Natal in Durban South Africa. I would like to ask for your permission to use one of your figures published in the Nature Journal for my masters thesis. The title of the article is : Insulin Signalling and The Regulation Of Glucose and Lipid Metabolism . I would like to reuse Figure 3 on pg 802 that depicts the regulation of glucose metabolism in the liver .

yours sincerely

Julia Hayangah

Saltiel, A. R. And Kahn , C. R.: Insulin Signalling And The Regulation Of Glucose And Lipid Metabolism.*Nature, December 2001; 414 (13): 799-806.http://www.nature.com/nature/journal/v414/n6865/index.html*

Alan Saltiel [saltiel@umich.edu]

In response to the message from Julia Hayangah, 08-10-2012

Julia Hayangah

Inbox

08 October 2012 03:18 PM

so granted.

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Julia Hayangah

Saltiel, A. R. And Kahn , C. R.: Insulin Signalling And The Regulation Of Glucose And Lipid Metabolism.*Nature, December 2001; 414 (13): 799-806.http://www.nature.com/nature/journal/v414/n6865/index.html*

Appendix 3- Staining Procedure

Histological staining

1. **Haemotoxylin and Eosin Stain**

Reagents

Reagent	Volume (ml) or Mass (g)
Haemotoxylin	1g
Distilled Water	1000ml
Potassium Alum	1 50g
Citric Acid	1g
Chloral Hydrate	50g
Sodium Iodate	0.2 g

Distilled water was added to a 3L conical flask and the following reagents were added into it namely haemotoxylin, potassium alum and sodium iodate. The mixture was then boiled for 5 minutes while constant stirring was maintained. After the five minutes the mixture was then cooled and filtered ready for use.

1% Acid Alcohol

Reagent	Volume (ml)
70% Alcohol	70
Concentrated Hydrochloric Acid	2
Distilled Water	28

The dilution process was done in a fume cupboard. 70 ml alcohol was mixed with 2mls of concentrated HCl and 28 ml distilled water.

Eosin Stain: Ready to use

Staining Procedure

The initial steps are mentioned in the Materials and Methods section (3.1) the temporary mounted slides are then used in the following procedure:

1. Stained with Mayers Haemotoxylin for 5 minutes.
2. Washed under running water until all excess stain was removed and the tissue appearedbluish.
3. The slides were then differentiated in acid alcohol.
4. Stained with Eosin for 10 minutes.
5. Washed under running tap water for 3 minutes.
6. Dehydration and clearing where performed as outlined in 3.1 and then permanently mounted.

2. Massons Trichrome Stain (MTS)

Reagents

a) Celestine Blue solution

Reagent	Volume (ml) or Mass (g)
Celestine blue B	2.5g
Ferric ammonium sulphate	25g
Glycerin	70ml
Distilled Water	50ml

Distilled water was used to dissolve the ferric ammonium sulphate. Continued stirring was maintained as the Celestine blue B was added and then the mixture was then boiled. The mixture was then cooled and glycerin was then added.

b) 1% acid solution

1% Acid alcohol was prepared as described in the previous section.

3. Acid Fuschin Solution

Reagent	Volume (ml) or Mass (g)
Acid Fuschin	0.5g
Glacial Acetic Acid	0.5ml
Distilled Water	100ml

Glacial acetic acid was placed in a beaker and a mixture of distilled water and Acid Fuschin was added to it and stirred.

4. Phosphomolybdic Acid solution

Reagent	Volume (ml) or Mass (g)
Phosohomolybdic Acid	1g
Distilled Water	100ml

1gram of the Phosohomolybdic acid was dissolved in 100ml of distilled water.

5. Methyl Blue Solution

Reagent	Volume (ml) or Mass (g)
Methyl Blue	2g
Glacial Acetic Acid	2.5ml
Distilled Water	100ml

2g of Methly Blue was dissolved in distilled water then added into the glacial acetic acid .

Procedure

The initial steps are mentioned in the Materials and Methods the temporary
Mounted slides are then used in the following procedure:

1. The nuclei were stained using Celestine blue for 5 minutes.
2. The slides were rinsed using distilled water.

3. The tissues were then immersed in Mayers Haemotoxylin for 5 minutes.

4. The slides were washed till they appeared bluish.

5. The tissues were differentiated using acid alcohol.

6. The slides were transferred to acid fuschin solution for 5 minutes.

7. The slides were rinsed using distilled water.

8. Slides were treated using phosphomolybdic acid for 5 minutes and then drained.

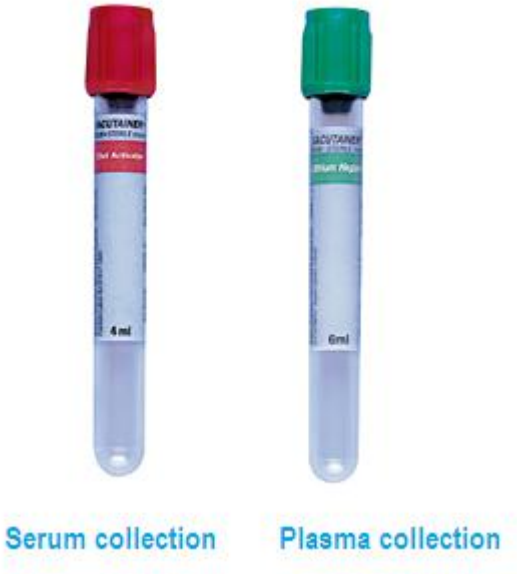
9. The slides were stained with methyl blue for 5 minutes.

10. The slides were rinsed using distilled water.

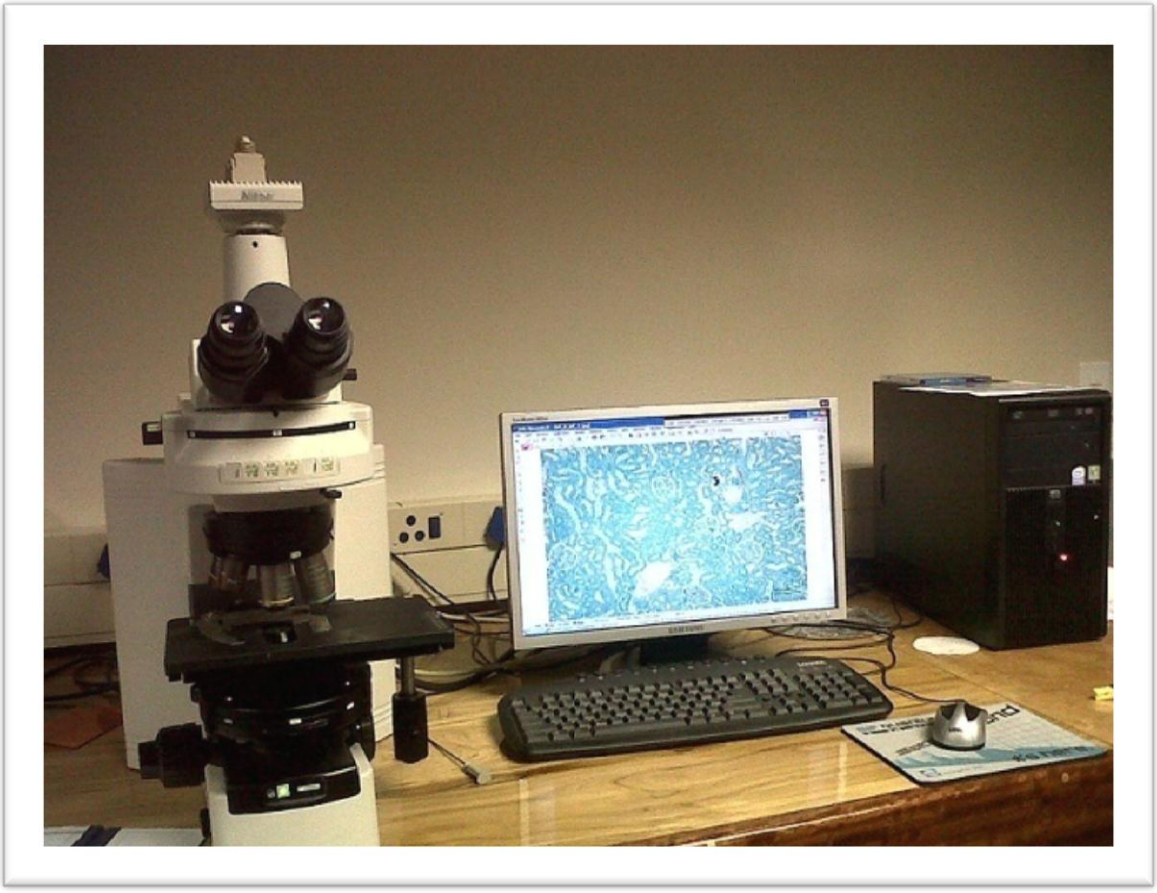
11. They were then treated with acetic acid for 2 minutes.

12. Dehydration and clearing where performed as outlined in 3.1 and then permanently mounted.

Appendix 4- Equipment used



Blood collection kits: Vacationers with red and green tops for serum and plasma collection



The Nikon Compound Light Microscope attached to a computer

Appendix 5- British Journal of Nutrition publication submitted

Hayangah, J.A., Ngubane S.P. and Owira PMO: Grapefruit juice improves glucose intolerance in streptozotocin-induced diabetes by suppressing hepatic gluconeogenesis

From: edoffice@nutsoc.org.uk [mailto:edoffice@nutsoc.org.uk]
Sent: 07 November 2012 06:28 PM
To: Peter Owira
Subject: BJN-2012-019123 Acknowledgement of new submission to the BJN

BJN-2012-019123
Grapefruit juice improves glucose intolerance in streptozotocin-induced diabetes by suppressing hepatic gluconeogenesis

Dear Dr. Owira,

Thank you for submitting the above paper to the British Journal of Nutrition. It has been accepted for the initial stage of the peer review process and is being considered for publication. You will be notified of our decision in due course.

The reference number of your manuscript is: BJN-2012-019123.

To check the status of the paper select the "Check Status" link at the following URL:

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Thank you for your interest in the British Journal of Nutrition.

Yours sincerely,

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