FRUCTOSE-FED STREPTOZOTOCIN-INJECTED RAT: AN ALTERNATIVE MODEL FOR TYPE 2 DIABETES

by

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Submitted in fulfillment of the academic requirements for the degree

MASTER OF SCIENCE

to the School of Biochemistry, Genetics and Microbiology
Faculty of Science and Agriculture
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As the candidate's supervisor, I have approved this dissertation for submission.

Supervisor: Dr M.S. Islam Signed_____ Date___ _____ 2011

PREFACE

The experimental work described in this thesis was carried out at the Department of Biochemistry under the School of Biochemistry, Genetics and Microbiology, Faculty of Science and Agriculture, University of KwaZulu-Natal, Westville Campus, from January 2010 to November 2011, under the supervision of Dr M.S. Islam.

These studies represent an original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

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

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

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DECLARATION 2 - PUBLICATIONS

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

Publication 1: *In press*
Fructose-fed streptozotocin-injected rat: An alternative model for type 2 diabetes.
Rachel Dorothy Wilson and Md. Shahidul Islam, **Pharmacological Reports**.

All laboratory work was conducted by Rachel Dorothy Wilson and where needed, assisted by Dr. MS Islam. The original manuscript was written and prepared by Rachel Dorothy Wilson and corrected, edited and proof-read by Dr. MS Islam.

Publication 2: *In press*
Book Chapter in *"Methods in Molecular Biology"* Textbook
"Experimentally induced rodent models of type 2 diabetes"
Md. Shahidul Islam and Rachel Dorothy Wilson, Human Press, New York, USA.

The book chapter was written by Rachel Dorothy Wilson in collaboration with Dr. MS Islam.

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DECLARATION 3 – PRESENTATIONS

DETAILS OF CONTRIBUTION TO PRESENTATIONS that form part and/or include research presented in this thesis.

Local presentations

Oral Presentation 1:

Fructose-fed streptozotocin-injected rat: An alternative model for type 2 diabetes.

School Research Day, 10 November, 2010. School of Biochemistry, Genetics and Microbiology, University of KwaZulu-Natal (Westville Campus), Durban 4000, South Africa.

Awarded *"Best Oral Presentation 2010"* from the School.

Oral Presentation 2:

Anti-diabetic effects of white mulberry leaf tea and L-carnitine in a newly developed rat model of type 2 diabetes.

Faculty Research Day, 31 October, 2011. Faculty of Science and Agriculture, University of KwaZulu-Natal (Westville Campus), Durban 4000, South Africa.

International presentation

Oral Presentation:

Fructose-fed streptozotocin-injected rat: An alternative model for type 2 diabetes. 21st World Diabetes Congress organized by International Diabetes Federation (IDF), 4-8 December, 2011, Dubai International Convention Center, Dubai, United Arab Emirates (UAE).

Awarded *travel and living grants by International Diabetes Federation (IDF) to present this work in the above-mentioned congress (prospective).*

Signed by student:

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ACKNOWLEDGEMENTS

Thanks be to God, for His everlasting love, guidance, grace and wisdom not only throughout the production of this thesis, but for everything that has been and ever will be. “Blessed are those who have discovered wisdom, those who have acquired understanding! Gaining her is more rewarding than silver, her yield is more valuable than gold. She is beyond the price of pearls, nothing you could covet is her equal. In her right hand is length of days; in her left hand, riches and honour. Her ways are filled with delight, her paths all lead to contentment.” (Proverbs 3:13-17)

Thank you to the National Research Foundation (NRF), South Africa and Competitive Research Grant from the Research Office, University of KwaZulu-Natal (Westville Campus) for generous funding of this project.

My most sincere gratitude and thanks go out to the following people:
To my supervisor, Dr MS Islam, for so many things: for inspiring my passion, for his continual motivation to be the best that I could be, his excellent work ethic, for teaching me everything he knows and passing on valuable skills to me, his insight, wealth of knowledge and expertise, guidance, assistance, patience, willingness to assist, organization and assurance throughout the study. Looking back from where I have came, I would not have been able to achieve it without him.

To my research group colleagues, Auwal Ibrahim, Dr Rahman and in particular Mitesh Indarjit for his assistance and companionship in 2011, and especially Andrew Mukundwa in 2010 and 2011, for his insight, companionship and assistance always.

To my other colleagues who have assisted me throughout the study: Nicolisha Narainpersad, Adhika Balgobind, Kovashnee Naicker, Aliscia Daniels for the office chatter and motivation, Blake Balcomb, for his friendship, great advice, assistance and insight, Karyn Pretorius for being a good friend who is always available to help out, Mele Duma for saving me a few times in a rush, Alain le Vieux for sharing the ups and downs of the journey and Marc Humphries for his support and guidance and willingness to help out whenever needed.

To the Biochemistry and Microbiology Department:
To Dr Mogie Singh, for allowing me to use her laboratory, Professor Mario Ariatti for his continued interest, support, faith and guidance throughout my entire university career, Professor Gupthar for advice and assistance, Dr Masola, Mr Hendricks and Patrick Govender, for their interest throughout my university career, to Nirasha Nundkumar for friendship and support and Dr Ade Olaniran, for his continued assistance and camaraderie throughout my university career. I send my special thanks to Professor Daniels for allowing me to utilize his histopathology laboratory and also to Shoohana Singh, for her technical expertise, assistance in the histopathological study and good nature throughout the study. To Linda Bester, David, Dennis and Ritta for absolutely everything they have assisted me with in the Biomedical Resource Center during the major portions of my project, your time and assistance is greatly acknowledged.

Finally, to all my friends, who without, I would not be the person I am today and have succeeded as far as I have, in particular:
Lesley Connolly, for everything over the last 16 years, thank you!
Jeffrey Timm, for being my continual support and always at hand to help whenever and without hesitation.
Lauren Dwyer and Lucy Sessions, for their interest, motivation and friendship throughout.
Nikhil Gulati for his insight, Dr. Fraser Pirie and Celestie Engelbrecht for inspiring me to take my passion further.

To my second family:
Luke Shannon, thank you for all your love always and everything you have done for me, and to the entire Shannon family, Paul, Karen, Warrick, Ceile and Joanie, including Reynard and Lynda White for all the hours, days, weeks and months of kindness, support, love, assistance and hours dedicated to helping me in the project. To my own family, Raphael, David and Biancca, thank you for all the help, laughter and assistance on the night shifts of the project, thanks to Joanne Long for helping out when needed, to my Dad, thank you for everything and all the hours of travel, and most of all, to my Mum, thank you for everything, for all the assistance, guidance, motivation, love and support.

ABSTRACT

The principal objective of this study was to develop an alternative non-genetic rat model for type 2 diabetes (T2D). Six-week-old male Sprague-Dawley rats (190.56 ± 23.60g) were randomly divided into six groups namely: Normal Control (NC), Diabetic/Streptozotocin Control (STZ), Fructose-10 (FR10+STZ), Fructose-20 (FR20+STZ), Fructose-30 (FR30+STZ) and Fructose-40 (FR40+STZ) and were fed a normal rat pellet diet *ad libitum* for 2 weeks. During this period, the two control groups received normal drinking water whilst the fructose groups received 10, 20, 30 and 40% fructose in drinking water *ad libitum* respectively. After two weeks of dietary manipulation, all groups except the NC group received a single injection (i.p.) of streptozotocin (STZ) (40mg/kg BW) dissolved in citrate buffer (pH 4.4). The NC group received only a vehicle buffer injection (i.p.). One week after the STZ injection, animals with non-fasting blood glucose >300 mg/dl were considered as diabetic. Three weeks after the STZ injection, the animals in FR20+STZ, FR30+STZ and FR40+STZ were eliminated from the study due to the severity of diabetes and the FR10+STZ group was selected for the remainder of the 11 weeks experimental period. The significantly ($p < 0.05$) higher fluid intake, blood glucose, serum lipids, liver glycogen, liver function enzymes and insulin resistance (HOMA-IR) and significantly ($p < 0.05$) lower body weight, oral glucose tolerance, number of pancreatic β-cells and pancreatic β-cell functions (HOMA-beta) of FR10 group demonstrate that the 10% fructose-fed followed by 40 mg/kg of BW STZ injected rat can be an excellent alternative model for T2D.

To validate this newly-developed model, an acute intervention trial study was conducted to investigate the anti-diabetic effects of L-Carnitine and white mulberry leaf tea extracts in the newly developed animal model of type 2 diabetes (T2D). Male Sprague-Dawley rats (mean BW 191.88g±16.40g) were randomly divided into 5 groups namely: Normal Control (NC), Diabetic/Streptozotocin control (FR10+STZ), Mulberry Tea Low (FR10+STZ+MTL, 0.25%), Mulberry Tea High (FR10+STZ+MTH, 0.5%), and L-Carnitine (FR10+STZ+CARN). In first three weeks, T2D was induced in all other groups except NC group by using above-mentioned procedure. Mulberry tea was supplied *ad libitum* and L-carnitine was administered to the FR10+STZ+CARN group at a concentration of 500mg/kg BW once daily during week 4-8 of the intervention trial. The FR10+STZ+CARN group had significantly ($p < 0.05$) lower total cholesterol, triglycerides, total proteins and fluid intake compared to the diabetic control (FR10+STZ). The NFBG non-significantly reduced in

FR10+STZ+CARN group compared to the FR10+STZ group, whereas MT did not. FR10+STZ+MTL had significantly higher serum triglycerides level compared to the NC group, and significantly higher HDL-cholesterol and fluid intake compared to the FR10+STZ group. FR10+STZ+CARN and FR10+STZ+MT groups had significantly lower total proteins compared to NC and FR10+STZ groups, but significantly lower albumin compared to NC group only. The data of the this section of the study suggest that CARN may be effective in normalizing lipid profiles rather than blood glucose in diabetic rats which may aid in the reversal of insulin resistance. On the other hand, MT used in this study did not display any significantly beneficial anti-diabetic effects at least in this experimental condition.

CONTENTS

ABBREVIATIONS

ADA	:	American Diabetes Association
ADP	:	Adenosine diphosphate
AGE	:	Advanced glycation end product
ALT	:	Alanine transaminase
AMPK	:	AMP-activated protein kinase
AST	:	Aspartate transaminase
ATP	:	Adenosine triphosphate
BG	:	Blood glucose
BW	:	Body weight
CARN	:	L-Carnitine
CAT	:	Carnitine-acetyltransferase
CCK-A	:	Cholecystokinin A
DBC	:	Diabetic Control
DIO	:	Diet-induced obesity
DNA	:	Deoxyribonucleic acid
DNJ	:	1-Deoxynorijirimycin
ELISA	:	Enzyme-linked immunosorbent assay
FBG	:	Fasting blood glucose
FFA	:	Free fatty acid
FPG	:	Fasting plasma glucose
FR	:	Fructose
G-6-P	:	Glucose-6-phosphate
GFAT	:	Glutamine:fructose-6-phosphate aminotransferase
GK	:	Goto Kakizaki rat
GlcNAc	:	N-acetylglucosamine
HDL	:	High density lipoprotein
HFCS	:	High fructose corn syrup
HK	:	Hexokinase

HOMA	:	Homeostasis model assessment
IFG	:	Impaired fasting glucose
IGT	:	Impaired glucose tolerance
IPGTT	:	Intraperitoneal glucose tolerance test
IRS	:	Insulin receptor substrates
IUGR	:	Intrauterine growth retardation
LDL	:	Low density lipoprotein
MODY	:	Maturity onset diabetes in young adults
MSG	:	Monosodium glutamate
MT	:	Mulberry tea
MTH	:	Mulberry tea high dose
MTL	:	Mulberry tea low dose
NA	:	Nicotinamide
NAD^+	:	Nicotinamide adenine dinucleotide
NADH	:	Nicotinamide adenine dinucleotide (reduced)
NC	:	Normal Control
NFBG	:	Non-fasting blood glucose
OGT	:	*O*-N-acetylglucosamine transferase
OGTT	:	Oral glucose tolerance test
OLETF	:	Otsuka Long Evans Tokushima Fatty Rat
PDH	:	Pyruvate dehydrogenase
Pdx-1	:	Pancreatic duodenal homeobox factor 1
PFK	:	Phosphofructokinase
ROS	:	Reactive oxygen species
SD	:	Standard deviation
SDT	:	Spontaneously diabetic Tori rat
SHR	:	Spontaneously hypertensive rats
SREBP	:	Sterol regulatory element binding protein
STZ	:	Streptozotocin
T1D	:	Type 1 diabetes
T2D	:	Type 2 diabetes

UDP	:	Uridine diphosphate
VLDL	:	Very low density lipoprotein
ZDF	:	Zucker diabetic fatty rat

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CHAPTER 1 General Introduction and Literature Review

1.1 Overview and global impact on diabetes

Type 2 diabetes mellitus is one of the most complex diseases to define in the medical world. Diabetic patients are varied from non-obese subjects with mild glucose intolerance to obese patients with moderate symptoms that are either acutely or chronically expressed (Nattrass and Bailey, 1999). Diabetes is thus a complex, heterogeneous disorder, caused as a result of a plethora of both environmental and genetic factors (Cnop *et al.*, 2005). Unlike Type 1 diabetes (T1D), where T1D is denoted as diminished insulin production, Type 2 diabetes (T2D) is seen as an insidious condition (Liu *et al.*, 2007). T2D is defined as impaired responses to insulin (insulin resistance) and progressive pancreatic β-cell dysfunction (Lin and Sun, 2010). Out of the total number of diabetic cases reported in Northern America, approximately 90-95% of them are Type 2 diabetics (Wing, 2008), with an estimated 20% of the population over the age of 65 suffering from T2D (Zimmet *et al.*, 2001).

Now-a-day diet and lifestyle choices tend toward high caloric intake and low physical activity, promoting the development of obesity. An increased risk of developing T2D has been linked to obesity through its association with diets of high caloric content (Marshall *et al.*, 1994). Furthermore, it is estimated that 80% of Type 2 diabetics are associated with obesity (Lin and Sun, 2010). From 1935 to 1996, levels of T2D have risen an astounding 765% (Basciano *et al.*, 2005).

High lipid levels in the blood stream seen in diabetics are also present in many obese individuals as fat secretes humoral factors including leptin, resistin and adiponectin that alter insulin sensitivity, leading to insulin resistance. This links obesity to a predisposition for the development of T2D (Trujillo and Scherer, 2006). This can be seen in the figures obtained from The International Obesity Task Force that estimated that there are approximately 300 million individuals suffering from obesity (Reuter, 2007); 150 million of which are suffering from T2D (Srinivasan and Ramarao, 2007). In 2000, 171 million people were reported to have T2D, (over 800 000 in South Africa), and this figure is predicted to rise to over 360 million by 2030 (with 1.2 million in South Africa) (WHO, 2010). This figure is alarming as T2D is the fourth major cause of death in South Africa (Joubert *et al.,* 2007) after HIV/AIDS, heart disease and respiratory infections. The socio-economic consequences of this disease are significant, as it is estimated that in 9 years (2006-2015), major world powers such as China

will lose over $558 billion from national income, due to diabetes and its complications (WHO, 2010).

Table 1: Projection of type 2 diabetic patients according to the World Health Organization (2010)

	2000	2030
World	171,000,000	366,000,000
South Africa	814,000	1,286,000

As mentioned above, T2D is classified according to two major pathogeneses presented as insulin resistance i.e. insulin insensitivity and impaired pancreatic β-cell function (DeFronzo, 1992). Insulin resistance is defined as an increase in insulin secretion in order to allow for a normal biological response from defective target tissues (Kural *et al*., 2007). Insulin resistance is an early feature of T2D largely linked to abdominal obesity, high intake of dietary carbohydrate with minimal physical activity, aging and several genetic factors (Stumvoll *et al.,* 2005; Kural *et al.*, 2007). Insulin insensitivity arises primarily from glucose storage target tissues such as skeletal muscle and the liver (Caro, 1990). Due to insulin resistance, pancreatic β-cells secrete abnormally high levels of insulin in order to control blood glucose levels, however overtime; hyperglycemia, hyperinsulinemia, pancreatic β-cell dysfunction and subsequent progressive pancreatic β-cell destruction occur (Stumvoll *et al.*, 2005; Liu *et al.*, 2007; Srinivasan *et al.*, 2005).

Insulin secretions occur in a biphasic fashion. First phase insulin secretions become impaired in type 2 diabetic patients, leading to a hyperglycaemic state. To compensate for hyperglycemia, a second, more pronounced insulin response occurs, which leads to hyperinsulinemia. As the disease progresses, even the second-phase insulin secretion response becomes impaired (Nattrass and Bailey, 1999). T2D is therefore noted as raised fasting blood glucose levels above that deemed to be normal as well as abnormally high postprandial blood glucose levels (Islam, 2011; Rees and Alcolado, 2005). Normal fasting blood glucose levels are below 100 mg/dl or less than 5.5 mmol/L, whilst impaired glucose tolerance is defined as mild hyperglycemia in the fasted state with blood glucose levels ranging between 120-140 mg/dl or 6.7-7.8 mmol/L. A full blown T2D state is defined as overt fasting hyperglycemia with blood glucose levels exceeding 140 mg/dl or greater than

7.8 mmol/L (De Fronzo, 1992). Whilst diagnostic characteristics defining T2D are well established, the mechanisms behind the onset of T2D remain largely obscure, although several mechanisms of action have already been reported. Many of these reported involve increased levels of non-esterified fatty acids, inflammatory cytokines, adipokines, glucotoxicity, lipotoxicity, mitochondrial dysfunction as well as amyloid formation resulting in pancreatic β-cell dysfunction (Stumvoll *et al*., 2005). The heterogeneity of this disease does not only stem from environmental factors, as there is a significant genetic component that influences the progression of this disease. Research in this area is limited, as only a few genes have been identified which play a role in the development of T2D. It is known that amongst identical twins there is a 90% concordance for the development of T2D (Marshall and Bangert, 2008). Abnormalities in genes such as glucokinase, calpain 10, potassium inward-rectifier 6.2, peroxisome proliferator-activated receptor γ (PPAR-γ) and insulin receptor substrate-1 (IRS-1) have been linked to the progression of T2D (Stumvoll *et al*., 2005).

1.2 Carbohydrate metabolism and biochemistry of insulin

Under normal carbohydrate metabolic conditions, pancreatic β-cells constantly produce insulin at a basal concentration irrespective of the blood glucose level. Following consumption of a carbohydrate-rich meal, complex carbohydrates are primarily metabolized by the salivary amylase in the mouth and broken down into disaccharide units. These are then hydrolytically split into monosaccharide units, largely comprising of glucose, fructose and galactose. These monosaccharides are then absorbed by the small intestinal mucosal cells and enter into the blood stream. Glucose is transported predominantly to the liver, where glucokinase, a rate-limiting enzyme of glycolysis responsible for glucose phosphorylation to glucose-6-phosphate, promotes glucose utilization (Figure 1).

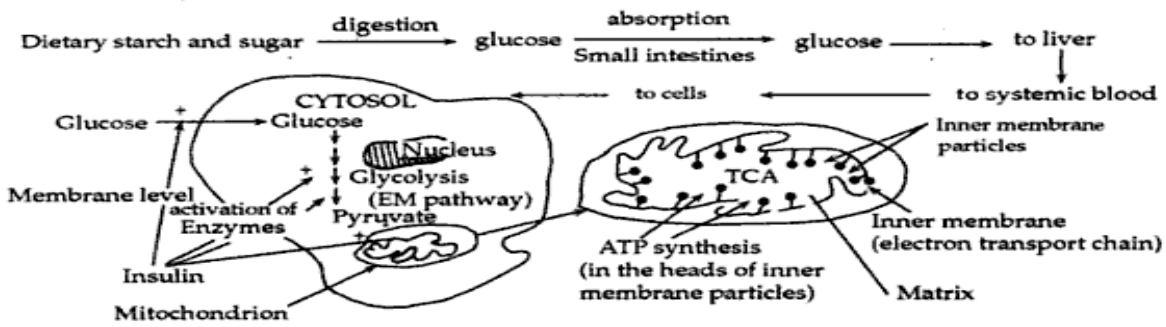

Figure 1: Overview of the cellular metabolism of carbohydrates (Copied without permission from Ramakrishnan *et al.*, 2001).

Glucose enters pancreatic β-cells via a GLUT-2 receptor and is metabolized by the rate-limiting enzyme, glucokinase, to glucose-6-phosphate. This then follows through the glycolytic pathway to form pyruvate, which enters into the mitochondria to generate energy in the form of ATP. This establishes an ATP/ADP ratio, which stimulates the opening of the ATP-sensitive potassium (K^+) channel. There is an efflux of potassium ions, resulting in the depolarisation of the cell. This allows for calcium ions (Ca^{2+}) to enter into the cell such that it is repolarised. Calcium ions exert an effect on proinsulin granules by modifying over 90% to the active form of insulin, that is then translocated to the plasma membrane of the pancreatic cells (Marshall and Bangert, 2008) (Figure 2).

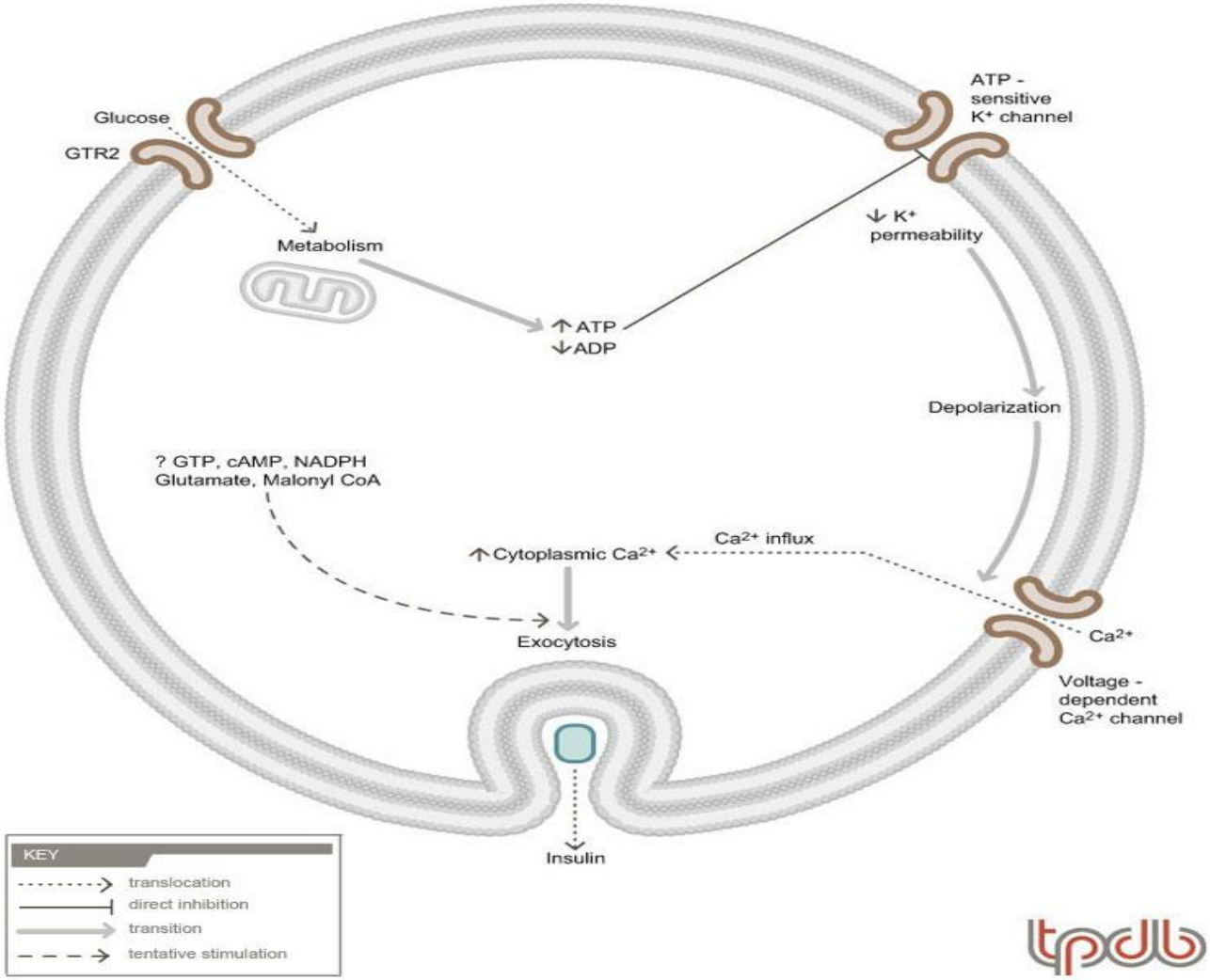

Figure 2: Mechanism of insulin secretion (Copied without permission from Wing, 2008) where GTR-2 = GLUT-receptor-2.

Upon elevation of the blood glucose levels, insulin stored in vacuoles is released into the blood stream, thereby regulating glucose uptake from the blood into skeletal muscle and adipose tissue (Figure 2) (Lin and Sun, 2010). For normal insulin function, it must bind correctly to its specific receptor, which allows for amplification of its signal via secondary-messenger pathways. These then allow for regulation and control of both cytoplasmic and genomic functions of insulin. Insulin signaling and transmission occurs through autophosphorylation of tyrosine residues within the receptor by the activation of insulin receptor tyrosine kinase activity. Several intracellular proteins are involved in this process and are known as insulin-receptor substrates (IRS). Dependent on the phosphorylation of these, the insulin signal is directed toward different metabolic pathways (Nattrass and Bailey, 1999). Insulin resistance is thus a primary hallmark feature of T2D, associated with a malfunction in the cells that respond to insulin (i.e. post-receptor) (Lin and Sun, 2010). Insulin serves to function as an antihyperglycaemic agent by several means including: reducing the production of gluconeogenic precursors such as lactate, alanine and glycerol, reducing hepatic gluconeogenic enzyme activity, reducing hepatic breakdown of glycogen (glycogenolysis) to glucose, which result in a lower hepatic glucose output and instead, a promotion of glucose storage in muscles as glycogen (Marshall and Bangert, 2008).

With lower blood glucose levels, the demand for insulin decreases and conversely stimulates the release of glucagon from the pancreatic α-cells, stimulating glycogen conversion back to glucose, particularly seen in fasting conditions (Lin and Sun, 2010).

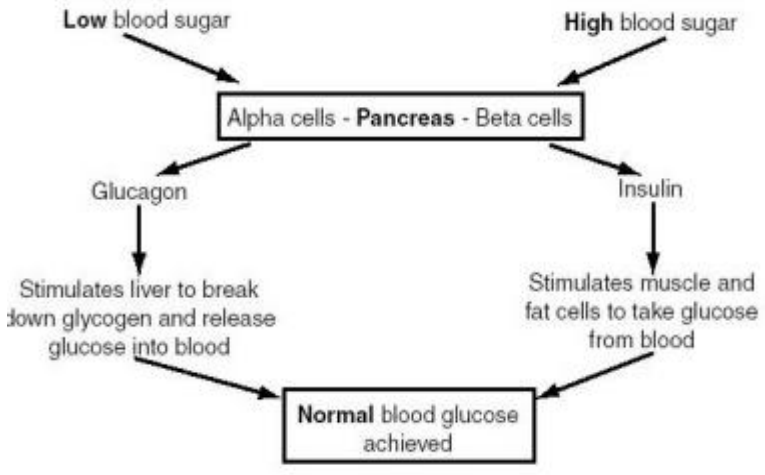

Figure 3: Roles of insulin and glucagon on carbohydrate metabolism and blood glucose levels (Copied without permission from Benardot, 2006).

1.3 Biochemical features of T2D

T2D leads to excessive urine production (polyuria), increased thirst (polydipsia), increased hunger (polyphagia), altered or affected vision (retinopathy), and major changes in energy metabolism such as hyperglycemia, hyperlipidemia and hyperinsulinemia (Lin and Sun, 2010). Fasting hyperinsulinemia, another early stage hallmark feature of T2D, results from a number of mechanisms including elevated levels of free fatty acids (FFAs) in circulation, which increase insulin secretion (Zhou *et al.*, 1999). Altered fatty acid metabolism has long been linked to insulin resistance (Shulman, 2000). The mechanism proposed for this (Figure 4) is known as the Randle cycle, which proposes that increases in fatty acids raise both intramitochondrial acetyl CoA/CoA and NADH/NAD^+ ratios. This leads to an inactivation of the enzyme pyruvate dehydrogenase, which increases intracellular concentrations of citrate. Citrate is a key-regulator of the glycolytic enzyme phosphofructokinase. Increases in citrate lead to a negative-feedback loop such that phosphofructokinase activity is inhibited. As phosphofructokinase is responsible for the conversion of glucose-6-phosphate to glucose-1,6-bisphosphate, its inhibition thus leads to an accumulation of glucose-6-phosphate. This in turn leads to an inhibition of hexokinase/glucokinase, responsible for the formation of glucose-6-phosphate from hexose sugars and glucose respectively. This means no phosphorylation of glucose occurs, resulting in accumulation of intracellular glucose by means of decrease glucose uptake by cells causing high blood sugar levels or hyperglycemia (Shulman, 2000). This sustained hyperglycemia results in increased insulin secretion in an attempt to lower blood glucose levels, causing hyperinsulinemia.

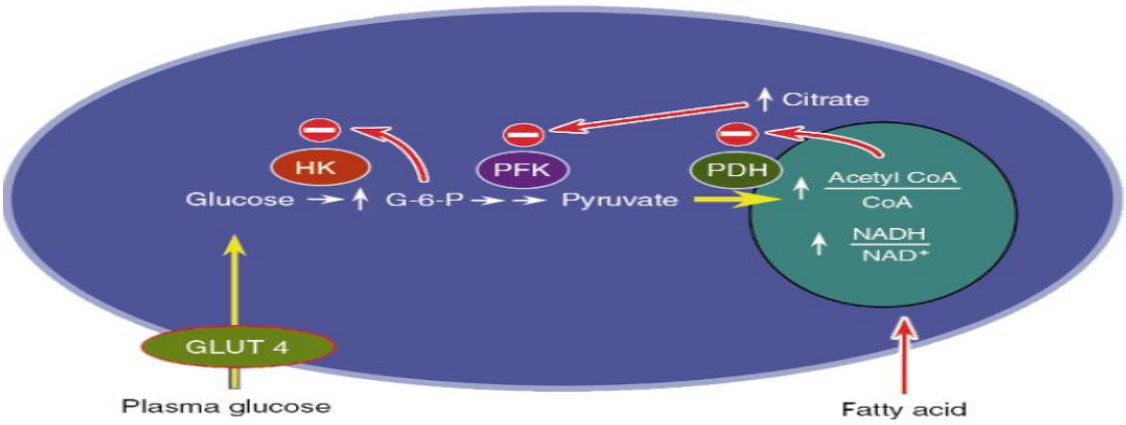

Figure 4: Mechanism of fatty acid induced insulin resistance (Copied without permission from Shulman, 2000, where HK = hexokinase, PFK = phosphofructokinase, PDH = pyruvate dehydrogenase, G-6-P = glucose-6-phosphate).

An alternative mechanism has also been proposed for fatty-acid induced insulin resistance in skeletal muscle (Figure 5). Alterations in fatty acid metabolism caused through a decrease in fatty acid metabolism result in increased intracellular concentrations of fatty acids and their metabolites such as fatty acyl CoA, diacylglycerol and ceramides. These metabolites are responsible for the activation of the serine/threonine kinase cascade, whereby serine or threonine residues become phosphorylated. When this occurs on the insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) sites, the ability of IRS-1/IRS-2 to further activate PI 3-kinase becomes diminished. This plays an important role in insulin signaling pathways and as a consequence leads to reduced glucose transport by GLUT-4 receptors. Non-entry of glucose into skeletal muscle results in increases in blood glucose levels causing hyperglycemia and insulin resistance as a result of diminished insulin signaling (Shulman, 2000).

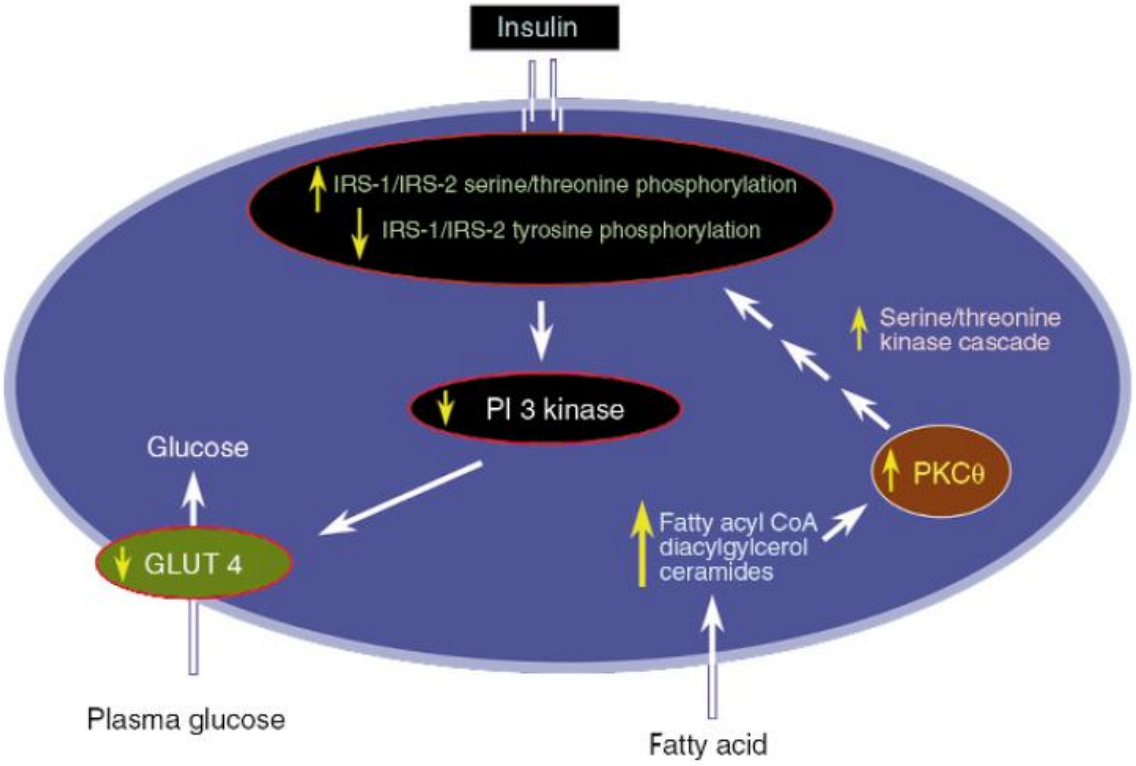

Figure 5: Alternative mechanism of fatty acid-induced insulin resistance (Copied without permission from Shulman, 2000)

Another characteristic feature of T2D, hyperglycemia, results from 3 main effects namely increased hepatic glucose production, lowered insulin secretion and finally an impairment in insulin action (insulin resistance) (Stumvoll *et al.*, 2005). As insulin resistance arises from impairment in insulin functioning in the liver, liver-related biochemical pathways including

gluconeogenesis, glycogenolysis, glycogen synthesis and glycolysis become defective. Insulin resistance in the liver thus leads to an inability to suppress the hepatic production of glucose. Fasting hyperglycemia (described earlier as blood glucose levels exceeding 140 mg/dl) in T2D patients has been attributed mainly due to increased hepatic glucose production (Caro, 1990). Hepatic glucose production in T2D is caused primarily as a result of increased gluconeogenesis, which most likely occurs due to a variety of mechanisms including those discussed above including chronically elevated levels of FFAs that activate gluconeogenic enzymes and elevated circulating levels of lactate, glycerol and alanine which are all important precursors for gluconeogenesis (Caro, 1990).

1.4 Current diabetes research

The World Health Organisation predicts that over 330 million people worldwide will have diabetes by 2030, and the percentages of national health care budgets to diabetes alone, are estimated at 5-10% (WHO, 2010). Of the diabetic cases, 90-95% of them suffer from Type 2 diabetes (T2D) (Chen and Wang, 2005). Due to its heterogeneous nature characterized by insulin resistance and pancreatic ß-cell dysfunction, it is essential to have appropriate means to study the molecular basis of the disease as well as advancing the knowledge on its pathogenesis including the environmental and genetic factors that contribute to the development of the disease. Furthermore, with the prevalence of T2D on the rise, drug development research and intensive studies into drug mechanistics have sharply increased; with the majority of this work being performed on animal models (Islam and Loots, 2009). Wall and Shani (2008) describe an animal model as "a living organism in which the phenomenon of interest can be studied and in which the phenomenon resembles that in the target animal in some respect." This allows one to study the biochemical mechanistics in an animal model and predict the outcome in another e.g. study a human disease state in an animal. Animal models are largely valuable to medicinal studies as biochemical pathways between mammals are largely conserved (Wall and Shani, 2008).

1.5 Materials used to induce diabetes for model development

1.5.1 Streptozotocin

Streptozotocin (STZ) is a natural antibiotic produced from the bacterial species *Streptomyces achromogenes* (Rees and Alcolado, 2005). It is a nitrosurea derivative and structural analogue of N-acetylglucosamine (GlcNAc) (Konrad *et al.,* 2001) that acts as a potent alkylating agent

that results in disrupted glucose transport, glucokinase activity as well as induction of multiple DNA strand breaks (Figure 6) (Bolzan and Bianchi., 2002).

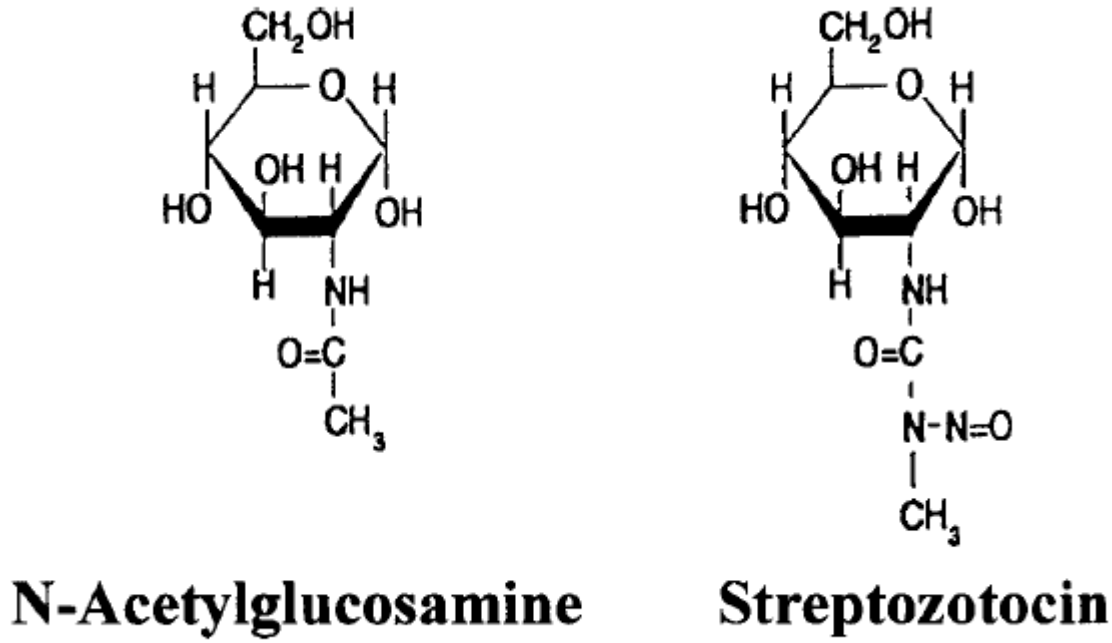

Figure 6: Structures of N-acetylglucosamine and STZ (Copied without permission from Konrad *et al.*, 2001).

Streptozotocin (STZ) is able to enter pancreatic β-cells through GLUT-2 receptors resulting in DNA damage through alkylation as well as cause the generation of toxic superoxide and hydroxyl radicals. These radicals result in the destruction of the pancreatic β-cells by necrotic mechanisms (Szkudelski, 2001). For this reason the use of STZ in diabetic research is commonly employed as it serves as a non-surgical method for the induction of hyperglycemia and progressive pancreatic β-cell destruction (Rees and Alcolado, 2005). Its use in induction of diabetes has been critical as it selectively targets pancreatic β-cells through the alkylation of their DNA, therefore being of major use in both T1D and T2D research (Zhang *et al.*, 2008). Streptozotocin (STZ) has also been found to be selective for the destruction of pancreatic β-cells as it inhibits the enzyme *O*-GlcNAc-selective-N-acetyl-β-D-glucosaminidase, which is responsible for the removal of *O*-GlcNAc from proteins. Protein glycosylation occurs when glucose is phosphorylated to glucose-6-phosphate and then transformed to fructose-6-phosphate under typical glycolytic processes. This is then converted to glucosamine-6-phosphate by glutamine:fructose-6-phosphate amidotransferase

(GFAT). This conversion provides precursors to UDP-GlcNAc, which is the necessary component in protein glycosylation. O-glycosylation in pancreatic β-cells takes place due to the enzyme *O*-GlcNAc transferase (OGT). This enzyme is expressed in substantially higher levels in the pancreatic β-cells than any other cell type in the body. STZ, thus, has been proposed to be selectively toxic to the pancreatic β-cells, leading to diabetic states (Konrad *et al.,* 2001).

The residual pancreatic β-cell mass that remains after STZ administration is estimated at 20%, however progressively increases to 50% due to pancreatic β-cell regenerative mechanisms (Masiello, 2006). Single high dose STZ injections result in massive pancreatic β-cell destruction, more characteristic of T1D, whereas low dose STZ injections cause only partial impairment to insulin secretory mechanisms seen in T2D (Srinivasan *et al.*, 2005).

1.5.2 Fructose

Fructose consumption in the modern world has vastly increased – not only from traditional intake of fruit, but largely as a result of being a constituent of many soda drinks and artificial sweeteners such as high fructose corn syrup (HFCS) (Benado *et al.*, 2004) (containing between 55-90% fructose) (Bray *et al.*, 2004) as well as from a high intake of sucrose (Basciano *et al.*, 2005). In 1970, it was estimated that the average individual consumption of fructose per annum was 0.5lb, which sharply rose to 62.4 lbs per annum in 1997 (Basciano *et al.*, 2005). Research indicates that the dietary intake of fructose in the United States of America today stands at approximately 10% of the total energy consumption, bearing in mind that only one third of the American population is deemed as "normal weight" (Kahn *et al.*, 2006). There is a great need for concern with regards to high-fructose intake and development of T2D. This is because studies done by Gross *et al.* (2004) revealed that nutrient consumption in the USA between 1909 and 1997 showed a significant correlation in the occurrence of T2D with carbohydrates, lipids, high-fructose corn syrups and calorie intake. However, taking into account the total calorie intake, protein and fat intake, only HFCS was associated with T2D.

Fructose has been labeled responsible largely for dietary-induced metabolic disturbances. Diets high in fructose cause weight gain, hyperlipidemia and hypertension in various animal models (Hwang *et al.*, 1987). Fructose has been shown to be more hyperlipidemic than both

glucose and starch through the causation of a dramatic increase in VLDL and LDL contributing to the increase in total cholesterol levels (Benado *et al.*, 2004).

Studies have shown that rodents fed dietary fructose at levels of 20% and greater were hyperlipidemic, with both serum total cholesterol and non-HDL cholesterol being significantly elevated ($p < 0.05$) (Benado *et al.*, 2004). This is of importance as diets containing 20% of the energy intake as fructose is approximately equal to the 90th percentile of fructose consumption in the American population.

Fructose is widely known for its potential to exacerbate hyperlipidemia. It has been suggested that because fructose enters the liver unrestrictedly without the need for a GLUT receptor, fructose metabolism is unique in that it can bypass several critical steps of the glycolytic pathway. This includes the need for insulin to activate glucokinase as well as the action of phosphofructokinase that, in the case of glucose, catalyses the conversion of glucose-6-phosphate to fructose-1,6-bisphosphate. Instead, fructose enters glycolysis or gluconeogenesis at the triosephosphate level in the liver (Benado *et al.*, 2004). This provides an important difference between glucose and fructose metabolism and their implications in diabetes. Glucose, as opposed to fructose, is therefore negatively regulated by phosphofructokinase, whereas fructose can unrestrictedly enter into the glycolytic pathway. This leads to the uncontrollable production of glucose by gluconeogenesis, as well as production of glycogen, pyruvate and lactate in glycolysis, leading to lactic acid formation involved in metabolic acidosis seen in diabetics.

Fructose is phosphorylated by fructokinase to yield fructose-1-phosphate, which is then split into two 3-carbon compounds namely glyceraldehyde and dihydroxyacetone phosphate. High concentrations of these provide excess acyl through the activation of pyruvate dehydrogenase and also glycerol molecules for triglyceride formation. This leads to esterification of fatty acids and their subsequent secretion as VLDL particles (Basciano *et al.*, 2005) and ultimately enhanced hepatic lipogenesis (See Figure 7). Furthermore, fructose does not appear to stimulate lipoprotein lipase (Kohen-Avramoglu *et al.,* 2003), which may result in reduced clearance of triglycerides from the plasma (Benado *et al.*, 2004). This has been a major contributing factor to the dyslipidemia noted in diabetic patients (Basciano *et al.*, 2005). In addition, studies have shown that fructose plays a key role in lipid synthesis as mice that followed a 7 day diet consisting of 60% fructose expressed an isoform of hepatic sterol regulatory element binding protein (SREBP) – a crucial transcription factor that regulates

both cholesterol and fatty acid synthesis. Fructose, unlike glucose, does not stimulate insulin nor does it stimulate leptin, which are both important for fat deposition in the body as well as energy intake regulation (Basciano *et al.*, 2005).

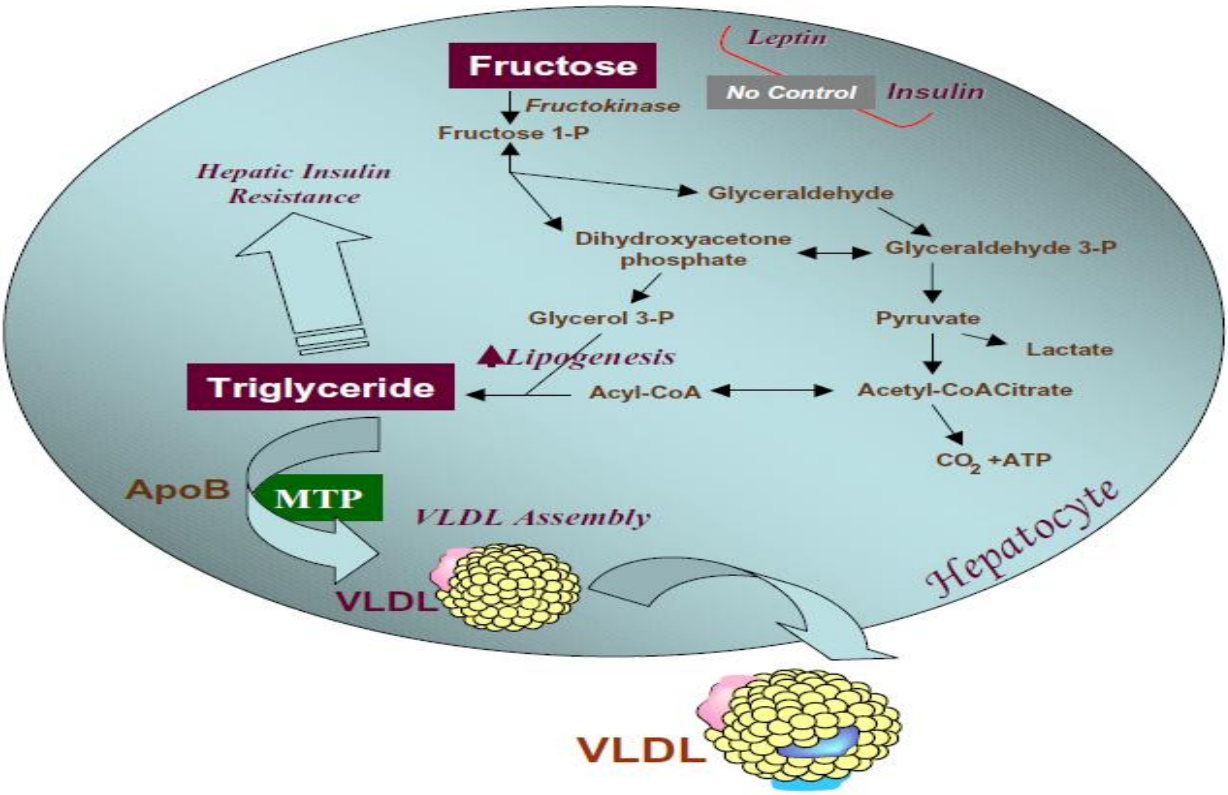

Figure 7: Diagrammatic representation of fructose metabolism (Copied without permission from Basciano *et al.*, 2005) ApoB = Apolipoprotein B, MTP = Microsomal triglyceride transfer protein, P = phosphate, VLDL= Very low density lipoprotein

A novel hypothesis proposes that fructose is capable of increasing levels of uric acid, which in turn, limit the availability of nitric oxide. Nitric oxide is required by insulin to stimulate the uptake of glucose from the blood stream, hence it is postulated that fructose is indirectly involved in promoting insulin resistance (Reuter, 2007). Studies in Australia at the Human Nutrition Research Center showed that fructose is also problematic as it is responsible for the depletion of chromium stores within the body. Chromium is important for diabetic patients as it is an essential mineral that aids the body in lowering blood glucose levels by acting in concert with GLUT receptors to facilitate entry of glucose into liver cells for further metabolism (Tondo, 2005). Furthermore, excessive fructose intake has been reported to alter protein oxidation biomarkers in the blood and enhance oxidative stress responses *in vivo* (Basciano *et al.*, 2005).

Whilst fructose is known to cause in insulin resistance, it is also indirectly linked to progressive pancreatic β-cell destruction. This is because insulin resistance, chronic hyperglycemia and hyperlipidemia as well as oxidative stress lead to activation of proinflammatory cytokines all induced by chronic fructose intake and subsequently trigger apoptotic mechanisms in pancreatic β-cells (Lin and Sun, 2010). Fructose has also been found to affect insulin sensitivity through its ability to reduce adiponectin responses; an adipocyte hormone that plays a vital role in insulin action and lipid homeostasis (Basciano *et al.*, 2005). Furthermore T2D has been linked to cardiovascular disease and elevated homocysteine levels are an important marker for cardiovascular disease. Interestingly, a study revealed that rats fed a fructose-rich diet had a 72% higher homocysteine level as opposed to the control, fed on regular chow (Basciano *et al.*, 2005), implying that fructose plays a role in the development of cardiovascular disease, a downstream complication of T2D.

1.5.3 Anti-diabetic drugs for model validation

1.5.3.1 Metformin

There are 7 major types of medication used in the treatment of diabetes namely insulin, sulfonylureas such as glibenclamide (commonly known as glyburide), biguanides such as metformin (commonly known as glucophage), alpha glucosidase inhibitors such as acarbose, thiazolidinediones such as pioglitazone and less commonly phenylalanine derivatives and meglitinides (Setter e*t al.,* 2003). Metformin, a biguanide, is derived from its parent compound, guanidine, originally found in the plant *Galega officinalis* (Goat's rue or French honeysuckle) (Bailey and Day, 1989).

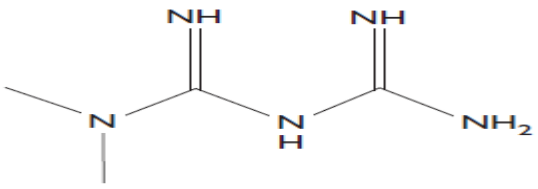

Figure 8: Chemical structure of metformin (Copied without permission from Setter *et al.,* 2003).

Today, metformin is the most widely used anti-diabetic drug in the world (Grisouard *et al.,* 2010) and has been a well-known drug for over half a century for the treatment of T2D (Howlett and Bailey, 1999). Metformin can be used in monotherapy or in conjunction with other treatments such as combinations with insulin or sulfonylureas (Campbell *et al.,* 1996).

Unlike sulfonylureas (e.g. glibenclamide), metformin does not increase insulin secretion (Howlett and Bailey, 1999), but rather improves hepatic sensitivity to insulin thereby aiding in the reduction of both fasting and postprandial blood glucose (Dunn and Peters, 1995). Metformin therefore does not result in hypoglycemia, nor does it cause weight gain, due to the fact that it does not increase insulin secretion as is the case with insulin and sulfonylureas (Campbell *et al.,* 1996). Furthermore, the two other major functions of metformin are that it reduces hepatic glucose production whilst also improving peripheral glucose uptake (Leverve e*t al.,* 2003).

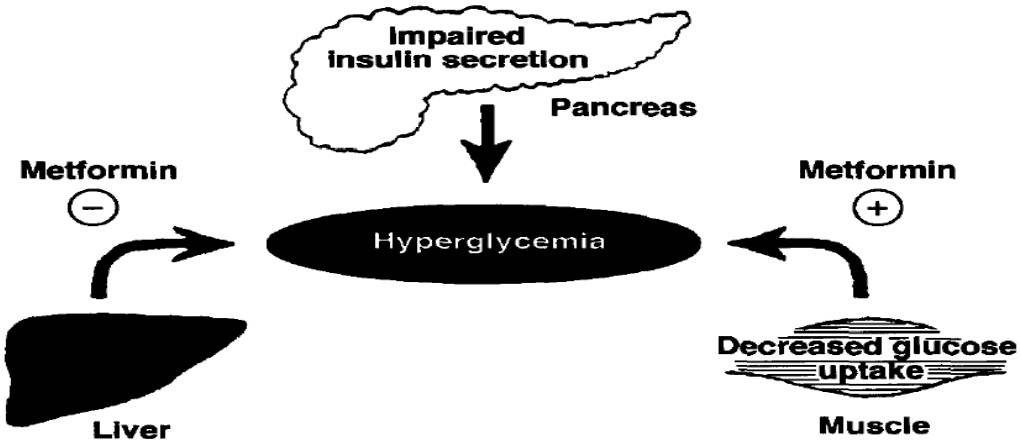

Figure 9: Mechanism of action of metfomin (Copied without permission from Defronzo e*t al.*, 1991).

The precise cellular mechanisms by which metformin acts are still poorly understood (Leverve *et al.,* 2003). One mechanism involves the ability of metformin to activate AMP-activated protein kinase (AMPK), which is a key regulatory enzyme in energy homeostasis within the body (Zhou *et al*., 2001). Literature indicates that metformin diminishes both cellular respiratory functions and mitochondrial function through the inhibition of the enzymatic activity of complex I in the respiratory chain both in skeletal muscle and hepatocytes (Detaille *et al.,* 2002). Grisouard *et al.* (2010) suggest that AMPK might also be activated in response to the inhibition of complex I. Metformin can thus serve to enhance glucose transport and glycolysis, as inhibition of the respiratory chain can be associated with an increase in the expression of glucose transporters and glycolytic enzymes. This serves to dramatically increase glucose utilization, thus lowering blood glucose levels (Grisouard e*t al.,* 2010). In animal studies, metformin increases tyrosine kinase activity, which is involved in insulin signaling (Del Prato e*t al.,* 1995). Enhancement of tyrosine kinase improves both skeletal glucose utilization and glycogen synthesis, further reducing blood glucose levels. Metformin has also been found to produce some beneficial results for lipid profiles. It has

been shown to successfully reduce total cholesterol, triglycerides and LDL cholesterol. It has however, shown no significant increases to high density lipoprotein (HDL) cholesterol levels (Trischetta *et al.,* 1992).

1.5.3.2 Glibenclamide

Glibenclamide (Figure 10) also known as glyburide (Langer *et al.,* 2000) is a second-generation sulphonylurea antidiabetic drug used in the treatment regiments of Type 2 diabetics (Caro, 1990). This drug is used to increase plasma concentrations of insulin, whilst decreasing plasma concentrations of both glucose and non-esterified fatty acids. It achieves the increase in insulin secretion by stimulating insulin release from the pancreatic β-cells. It is, however, universally accepted that glibenclamide is not effective if pancreatic β-cells are non-functional or damaged to a large degree, as research has shown in both animal models and human clinical trials (Musbah and Furman, 1980; Stocks *et al.,* 1988). Glibenclamide also produces extrapancreatic effects by improving the effect of insulin on target tissues such as the liver (Caro, 1990, Tanira and Furman, 1999). It has been shown to enhance the action of insulin (increase insulin sensitivity) in the liver and concurrently decreases gluconeogenic activity. It achieves its hypoglycaemic effects by several mechanisms: increasing glycogen synthesis by stimulating glycogen synthase, inhibiting glycogen catabolism (glycogenolysis) by reducing phosphorylase α activity and finally suppressing gluconeogenesis, increasing metabolic pathway flux to glycolysis by decreasing A-kinase activity resulting in increased fructose 2,6-bisphosphate – a key regulator in carbohydrate metabolism (Caro, 1990).

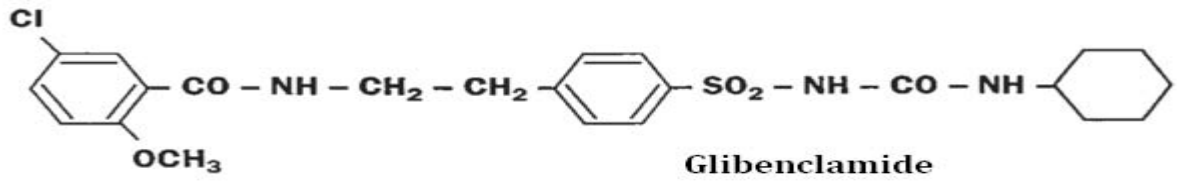

Figure 10: Structure of Glibenclamide (Copied without permission from Nattrass and Bailey, 1999)

In vivo studies by McGuinness and Cherrington (1990) determined the ability of glibenclamide to enhance insulin's opposing effect on the gluconeogenic hormone, glucagon. They found that in the absence of insulin (as seen in T1D patients), glibenclamide could not diminish the action of glucagon. In the presence of sufficient insulin, however, glibenclamide decreased both hepatic glucose production and phosphorylase α (a key enzyme involved in the breakdown of glycogen into individual glucose units) by 40% and 50% respectively

(McGuinness and Cherrington, 1990). This is important to note as it shows that glibenclamide is not a direct inhibitor of glucagon, but rather improves the action of insulin.

In vitro studies performed by Davidson and Sladen (1987) measured the catabolism of prelabeled glycogen and anabolism of glycogen through the incorporation of [^{14}C] glucose. Glibenclamide significantly reduced glycogen breakdown and instead, favoured its synthesis by stimulation of glycogen synthase. Glibenclamide also activates other major biochemical pathways such as the glycolytic pathway by enhancing the action of a major rate-limiting enzyme of this pathway, glucokinase, which phosphorylates glucose to glucose-6-phosphate. This thereby commits glucose to be metabolized by the liver to either generate energy or in excess amounts, be stored as glycogen in muscle or fat in adipose tissue. Further evidence of glibenclamide action comes from the compound fructose-2,6-bisphosphate (F-2,6-P_2), which can alter major rate-limiting enzymes of glycolysis and gluconeogenesis i.e. phosphofructokinase and fructose 1,6-bisphosphatase respectively as seen below (Caro, 1990).

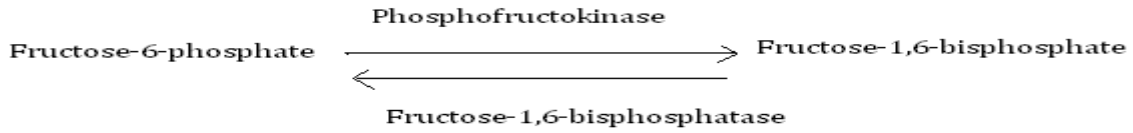

Figure 11: Biochemical reaction and enzymes in fructose derivative reactions (image self drawn)

This compound in higher concentrations activates phosphofructokinase and hence glycolysis. Interestingly, diabetic animals possess lower concentrations of F-2,6-P_2 in the liver, which may provide an important reason for increased gluconeogenesis and decreased glycolysis seen in T2D. Glibenclamide has been found to enhance F-2,6-P_2 formation in a dose-dependent manner. Deeper insight reveals that biochemically, formation of F-2,6-P_2 is controlled by a bifunctional enzyme (Fructose-6-phosphate-2-kinase-fructose-2,6-bisphosphatase) (Caro, 1990).

1.6 Overview of current animal models of T2D

1.6.1 Genetic models of T2D

Genetic models for T2D research have widely been employed over the last few decades. Some of the available models include the Zucker diabetic fatty (ZDF) rat, Goto Kakizaki

(GK) rats, Otsuka Long Evans Tokushima Fatty (OLETF) rats, spontaneously diabetic Tori (SDT) rats as well as genetically developed *db/db*$^{+/+}$ (Islam and Loots, 2009). Several knock-out models in mice have been developed and present with molecular pathologies of the disease yet often fail to exhibit exact symptoms of T2D (Nakamura *et al*., 2006). Advantages of these models, however, include allowing precise areas of pathogenesis to be studies and presenting with symptoms ranging from insulin resistance to reduced pancreatic β-cell mass (Islam and Loots, 2009). The disadvantages associated with these models are that they require high levels of maintenance and thus are high in cost. However, amongst researchers particularly in developing countries, these models are not widely available (Nakamura *et al*., 2006).

There are several knock-out gene models that exist. These models strive to delete critical genes, that without them, diabetic symptoms arise. For instance, animals lacking pancreatic duodenal homeobox factor 1 (Pdx-1) lack the essential transcription factor that is required for normal pancreatic development (Masiello, 2006). It plays an important role in pancreatic β-cell differentiation and regulation of critical pancreatic β-cell genes such as those for insulin and glucokinase. This results in congenital diabetes, often associated with maturity onset diabetes in young adults (MODY) as well as typical T2D (Masiello, 2006). This is a typical feature of the Zucker Diabetic Fatty (ZDF) rat (Chen and Wang, 2005). The Zucker diabetic fatty rat was developed from a diabetic trait originating from Zucker rats in the USA between 1974 - 1975. Animals with a diabetic lineage were then identified, leading to the development of a Zucker Diabetic Fatty rat inbred line being developed in 1985. It was only in 1991 that this model was developed as a genetic model for the study of diabetes (Chen and Wang, 2005). This rat also contains a genetic defect in the leptin receptor and hence the rats develop extreme obesity (Chen and Wang, 2005; Masiello, 2006), hyperglycemia and hyperlipidemia (Chen and Wang, 2005; Peterson *et al.,* 1990). These are known as fa/fa rats as they are homozygous for non-functional leptin receptors. Rats that are homozygous dominant (+/+) in nature and heterozygous (fa/+) remain lean and normoglycaemic.

The obesity seen in fa/fa rats leads to insulin resistance, however these rats can compensate through an increase in pancreatic β-cell mass leading to increased insulin secretion (Masiello, 2006). This model has proved to be highly advantageous for the study of T2D, as it allows for the progressive induction of the prediabetic state through to the diabetic state. Hyperglycemia is developed within 7 weeks of age, and by 12 weeks, all obese rats are fully diabetic (Chen and Wang, 2005). In addition, GLUT-4, the protein responsible for insulin-stimulated glucose

transport into skeletal muscle and adipose tissue (Patti and Kahn, 1996), is diminished by up to 55% in this model (Slieker *et al.,* 1992).

Other popular rodent model used for diabetic studies is the Otsuka Long-Evans Tokushima Fatty Rat (OLETF). This model was developed in 1984 and displayed characteristics of polyuria, polyphagia and polydipsia. Other characteristics include mild obesity and unique to this model is the delayed onset of hyperglycemia with its induction being noted only after 18 weeks of age. This model displays a chronic occurrence of the disease state and hence is ideal for long term studies involving therapeutic drug intervention trials (Kawano *et al.,* 1994). The basis for this model relies on the knockout of several recessive genes. One such gene is the cholecystokinin A (CCK-A) receptor gene, resulting in a diminished ability to determine satiety after meal consumption, leading to increases in meal size and later, obesity. Gene deficits also exist in the hypothalamus signaling pathways of neuropeptide Y (Bi and Moran, 2002). Hypertriglyceridemia and decreased GLUT-4 receptors also contribute to the heterogeneity of the diabetic pathogenesis of these animals (Chen and Wang, 2005).

1.6.2 Experimentally induced rodent models of T2D

Non-spontaneous or non-genetic models are so-named as they make use of rodent strains that under normal conditions are not diabetic (Islam and Loots, 2009). They are much cheaper in cost compared to genetic diabetic strains and are far more widely available to researchers worldwide. Experimentally induced models are further subdivided into the following categories: those induced by chemical means and those that involve dietary manipulation and some include a combination of these two. Besides the cost factor, these models have become highly popular in recent years due to the resemblance of the pathogenesis to the biochemical profile of diabetic humans, although no one single model clearly shows all pathogeneses and symptoms of T2D. However, in recent years, rodent model development has come a long way to produce models that resemble the two major pathogeneses of T2D – these being firstly insulin resistance and secondly, overtime, the development of partial pancreatic β-cell dysfunction.

1.6.3 Diet induced rodent models of T2D

1.6.3.1 Fat fed models

The most commonly used fat fed mouse model is the Diet Induced Obese (DIO) model using male C57BL/6J mice. The development of obesity is subjective to the amount of fat added to

the diet, with high fat diets used to induce obesity, whilst low fat diets are unable to induce obesity. An important consideration when using fat fed rodents to induce insulin resistance is the initiation of fat feeding in diets at a young age between 6-8 weeks, as this has been shown to be most effective to develop obesity (Reuter, 2007). These high fat fed male C57BL/6J mice are typically fed a diet containing 40-60% calories from fat, approximately 8 times higher fat content than that of control mice for 8-16 weeks, although diabetic features can be noted after just 4 weeks. The longer duration of fat-feeding enhances the features of insulin resistance, impaired glucose tolerance revealing elevated serum insulin and glucose, abnormal lipid profiling and hyperglycemia in a mild to modest form; the degree to which is dependent on the duration of fat feeding and the type of fat (Reuter, 2007; Winzel and Ahrén, 2004). Pancreatic islet dysfunction is also noted in this model, which is an essential pathogenesis for T2D (Reuter, 2007). The DIO rat model most popularly uses outbred Sprague-Dawley rats as it has remarkable sensitivity to high fat diets to induce insulin resistance and diabetes as opposed to other rat strains (Chang *et al.,* 1990; Reuter, 2007). Overtime symptoms of hypertriglyceridemia, hyperglycemia and hyperinsulinemia develop, leading to T2D. The major disadvantage of this model is the long duration of time required to induce insulin resistance and T2D (Srinivasan and Ramarao 2007), which is not suitable for many researchers, as this increases the cost of the experiment.

1.6.3.2 Fructose fed rodents

Research indicates that palatability of diets is an important factor in dietary choices, however sweet-tasting diets such as high fructose diets enhancing polyphagia and weight gain leading to obesity and insulin resistance (Gerrits and Tsalikian, 1993). Fructose-fed rats have drawn many associations to the typical American diet which constitutes approximately 10% of the dietary energy intake primarily from artificial sweeteners, soda drinks and their content of high fructose corn syrup (Benado e*t al.,* 2004). Fructose fed rat models target the diabetic profile at the lipid level. Hyperlipidemia is directly linked to the development of insulin resistance, the first major pathogenesis of T2D (Barnes *et al.,* 2009; Trujillo *et al*., 2006). Research indicates that fructose is more hyperlipidemic than both glucose and starch due to its unique metabolism (Benado *et al*., 2004). High-fructose diets have been shown to significantly increase total cholesterol levels through increases in very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) in rats.

Zavaroni (1980) and Tobey (1982) first demonstrated that fructose feeding in rats induced symptoms of hypertriglyceridemia, hyperinsulinemia and insulin resistance. To develop these pathogeneses, calorie intake in diets contained between 35-72% fructose or 10-15% fructose solutions in drinking water (Dai *et al*., 1994) *ad libitum* for 2-12 weeks. They demonstrated that rats fed a drinking solution of 5-10% fructose showed dose-dependent increases in fluid intake, as commonly seen in type 2 diabetics as polydipsia. Furthermore over the 14 week period, these rats experienced progressive weight gain. Their experiment also revealed feeding a 10% fructose solution (the equivalent of approximately 52.5% calories in a diet) in drinking water for 1 week or more was the most suitable model for developing a common secondary complication of T2D known as hypertension with increases in systolic blood pressure of 20-25mmHg (Dai e*t al.,* 1994).

In a study conducted by Benado *et al*. (2004), varying concentrations of fructose were analysed for their effects on lipid levels in normal rats. Their diet consisted of both fat and carbohydrate levels reflecting those typically seen in an average American diet. The fructose levels varied between 0%, 10%, 20% and 45% fructose. Although 10% fructose intake is deemed equivalent to that of the typical American intake, their results indicated no significant difference in total cholesterol, non-HDL cholesterol and triglycerides in 10%-fructose-fed rats, yet significant differences in the 20% and 45% groups when compared to the control (0% fructose). A significant mean body weight gain was only, however, noted in the 45%-fructose-fed group. The conclusions drawn indicate that fructose consumption at "normal" levels i.e. 10% of energy intake does not produce any adverse effects. However, this was not tested in a diabetic rat model.

1.6.4 Chemically induced rodent models of T2D

1.6.4.1 Adult Alloxan/Streptozotocin rodents

Alloxan was the first chemical inducer of T2D used in 1943 by Goldner and Gomori (Goldner and Gomori, 1943). Alloxan is a uric acid derivative that selectively destroys pancreatic β-cells by oxidative stress mechanisms (Rerup, 1970). Streptozotocin (STZ), on the other hand, is a natural antibiotic produced by the bacterial species *Streptomyces achromogenes* (Rees and Alcolado, 2005). It is a structural analogue of N-acetylglucosamine that acts as a potent alkylating agent that results in disrupted glucose transport, glucokinase activity as well as the induction of multiple DNA strand breaks (Bolzan and Bianchi, 2002). Various combinations over the decades have been used to develop models for diabetic

research, including single high dose STZ injection, multiple low dose STZ injections or in combination with high fat diets. It is, however known that, single high dose STZ injections higher than 60 mg/kg body weight (BW) result in massive pancreatic β-cell destruction, more characteristic of T1D, whereas low dose STZ injections between 40-55 mg/kg BW cause only partial impairment to insulin secretory mechanisms seen in T2D (Srinivasan *et al.*, 2005). Dosages lower than 35 mg/kg BW in rats fed a normal commercial diet fail to elicit any hyperglycaemic effect (Srinivasan *et al*., 2005). These compounds can be injected either intravenously (i.v.) or intraperitoneally (i.p) at dose concentrations between 35-65 mg/kg BW STZ in rats, 100-200 mg/kg BW STZ in mice and in the case of alloxan, 40-200 mg/kg BW in rats and 50-200 mg/kg BW in mice (Islam and Loots, 2009).

1.6.4.2 Neonatal Alloxan/Streptozotocin rodents

Neonatal alloxan and STZ diabetic models have been employed since the mid 1970's. A decade thereafter, several attempts at refining this model have been made, most successfully using doses of STZ between 25 mg/kg BW but less than 50 mg/kg BW in male spontaneously hypertensive rats (SHR) (Iwase *et al.,* 1986). In these models, STZ is most commonly delivered intraperitoneally to neonatal rats 2 days after birth. Initially until the 4th week, non-fasting blood glucose levels appear normal to moderately hyperglycaemic. As adulthood approaches, hyperglycemia progressively develops, leading to adult-onset T2D (Giddings *et al.,* 1985). Important to note are the discrepancies of these models when using different rodent strains. Two days old male Wistar rats have been shown to develop diabetes only when injected with significantly higher doses of STZ (90 mg/kg BW i.p) (Shinde and Goyal, 2003), yet Fischer 344 rats developed a stable and optimal diabetic state when injected intraperitoneally at a dose of 80 mg/kg BW. In both adult and neonatal alloxan/STZ models, features of hyperglycemia and glucose intolerance develop, however many are not validated by anti-diabetic drugs and thus limit their suitability as a model for T2D (Islam and Loots, 2009).

1.6.4 3 Monosodium glutamate induced models

Monosodium glutamate (MSG) is a sodium salt of glutamic acid and is frequently used as a flavour enhancer (Jinap and Hajeb, 2010). Studies of the effects of subcutaneous injections of MSG on neonatal rats began in the 1970's where the preliminary results showed detrimental effects on rat growth, brain and reproductive function as well as impaired glucose tolerance (Lengvari, 1977). In the late 90's numerous studies revealed the development of central

obesity and hyperinsulinemia in MSG treated rats (Ribeiro *et al*., 1997; Morrison *et al.,* 2007).

Iwase *et al.,* (1998) reported significantly higher serum triglycerides in 4 mg/kg BW MSG-treated rats as compared to control rats, however total cholesterol results remained statistically insignificant. Fourteen month old female SHR rats that were neonatally treated with 4 mg/kg BW MSG were also reported to have significantly elevated glycated haemoglobin, mesenteric fat and body weight, serum blood glucose and insulin as well as elevated systolic blood pressure.

Nagata *et al*., (2006) developed a novel ICR mouse model for T2D induced by MSG. Subcutaneous MSG injections (2 mg/kg BW) were delivered on the day of birth until the 4th day of age in both male and female mice, whilst normal saline was injected accordingly to control mice. Their results revealed a significantly higher body weight in both sexes compared to the respective controls after 7 weeks of age, however no significant differences were noted between food and fluid intake compared to the control groups, indicating the absence of polyphagia and polydipsia and concluding that obesity was not caused through polyphagia. These two characteristic features, however, are common symptoms of the diabetic condition. Male-MSG-treated mice developed glucosuria after 8 weeks, whilst females only developed this symptom after 19 weeks. After 12 weeks of age, the male mice demonstrated significantly impaired glucose tolerance and insulin resistance indicating a diabetic state. Furthermore the male mice demonstrated an abnormal liver enzyme profile, which is indicative of hepatic insulin resistance. Disadvantageously, this model developed small liver tumours, which could interfere in the interpretation of results when being used as a model for T2D. In the development of this model it was also shown that male mice serve as a better model as the females appear to be less susceptible to the detrimental effects of MSG.

Morrison *et al*., (2007) injected MSG at a dose of 4g/kg BW intraperitoneally from the first day of birth until the 7th day. Their results revealed central obesity in the MSG-treated rats as well as all rats over the age of 65 weeks having developed cataracts, a common complication of T2D. After 32 weeks of age, no significant differences were noted in glucose tolerance tests compared to control rats, however differences were noted after 65 weeks between the two groups. Whilst this comparatively new model for T2D offers both the major and minor pathogeneses of T2D, it is limited in its use as it has not been validated by anti-diabetic drugs

and similarly to the intrauterine growth retardation (IUGR) model, the long induction time of the diabetic state is unfavourable (Islam and Loots, 2009).

1.6.5 Combined rodent models of T2D

1.6.5.1 Fat-fed streptozotocin rodents

Diet induced diabetes was first described in 1947 by Houssay and Martinez. The fat-fed STZ-injected rat makes use of dietary manipulation to induce insulin resistance and STZ to induce partial pancreatic β-cell dysfunction. This model has a major advantage over genetic models in that it replicates the natural disease history and pattern as well as producing several characteristics that parallel the human condition (Chen and Wang 2005). In 2000, Reed *et al.* were the first to develop the fat-fed-STZ-injected rat drawing on the rationale that Sprague-Dawley rats develop both insulin resistance and hyperinsulinemia, but not hyperglycemia when fed a high-fat diet. The use of STZ caused a subsequent decline in plasma insulin concentrations leading to hyperglycemia and T2D. These rats were fed a diet consisting of 40% fat, 41% carbohydrate and 18% protein which was continued throughout the entire experimental period. After 2 weeks of feeding, the rats were injected with 50 mg/kg BW STZ.

A similar, but modified attempt at this model of T2D was done by Srinivasan *et al.* (2005). In their modifications, the high-fat diet contained 58% fat, 17% carbohydrates and 25% protein and was similarly supplied *ad libitum* throughout the entire experimental period. After the 2 week period of dietary manipulation, the rats received a 35 mg/kg BW injection intraperitoneally of STZ. This resulted in frank hyperglycemia, significantly elevated total serum cholesterol and serum triglycerides. Significantly decreased serum insulin compared to rats fed only a high-fat diet was also noted, yet no significant differences were noted between rats injected with STZ and fed a normal rat pellet diet. Advantageously this model was also sensitive to anti-diabetic drugs confirming its suitability as a model for T2D. Whilst it appears to be a good choice for use as a rodent model for T2D, several critical biochemical parameters have not been tested, including liver function enzymes, liver glycogen, pancreatic histopathology and serum creatinine (a measure of kidney function). Although the model remained stable over a 10 week experimental period, further biochemical data are required to enhance its suitability to long-term studies of diabetic complications such as nephropathy, neuropathy, retinopathy and cardiomyopathy.

Further modifications have been reported by Zhang *et al.* in 2008 that utilized a combination high-fat diet with multiple low-dose STZ injections. Their high-fat diet consisted of 22% fat, 48% carbohydrates and 20% protein. The rats were intraperitonteally injected with STZ twice at a dose of 30 mg/kg BW, with a 1 week period between injections. The high-fat-fed diet was supplied for a duration of 4 weeks. In contrast to Srinivasan's model, this model demonstrated both lower total serum cholesterol and serum triglycerides compared to the high-fat-diet control group, and only demonstrated significantly elevated fasting blood glucose compared to the normal rat pellet fed controls.

1.6.5.2 Streptozotocin-nicotinamide models

Nicotinamide (NA) is the amide derivative of nicotinic acid and is used as a therapeutic agent in diabetic research. It is a potent scavenger of free radicals and provides a component of the coenzyme nicotinamide adenine dinucleotide (NAD). Nicotinamide (NA) in high doses has been noted to have a protective function on pancreatic β-cell survival against both exogenous toxic agents as well as from immune responses (Knip *et al*., 2000). This can diminish the toxic effects exerted by STZ on pancreatic β-cells, with extremely high doses (350 mg/kg bw) having shown to fully prevent STZ-induced damage (Masiello *et al*., 1998). STZ induces DNA damage in pancreatic β-cells and activates the enzyme poly(ADP-ribose) synthetase. Nicotinamide (NA) inhibits this enzyme, thereby reducing apoptotic levels within the damaged pancreatic β-cells, thereby promoting pancreatic β-cell survival (Masiello *et al*., 1998).

The STZ-NA model was originally developed by Masiello *et al.* in 1998 and later, Nakamura *et al.* (2006) adapted this to resemble a non-obese non-genetic model of T2D in mice. Westernised diets tend to be higher in calorie content and thus pose a new set of problems in terms of metabolic disturbances. It is widely known that T2D patients show signs of insulin resistance, yet many non-obese patients of T2D are showing signs of inadequate insulin secretion, as opposed to insulin resistance (Nakamura *et al*., 2006). The STZ-NA models can therefore provide many similarities to type 2 diabetics that are non-obese (Nakamura *et al.*, 2006). This model is characterized by normal weight gain and insulin secretion despite a slightly altered ability to produce insulin from pancreatic β-cells. Furthermore it shows slightly altered fasting blood glucose levels and only mild glucose intolerance (Nakamura *et al.*, 2006). This model is advantageous in comparison to other non-genetic T2D models as it

can be quickly developed in a 5 week time period thus also allowing for experimental costs to be reduced (Nakamura *et al*., 2006).

The basis for this model relies on the theory that STZ-induced DNA damage stimulates DNA repairs mechanisms that consume large quantities of nicotinamide dinucleotide (NAD). This can be supplemented by consumption of NA. The NA serves as a partial protector against excessive pancreatic β-cell damage caused by STZ. Masiello *et al.* (1998) developed this model using a dose of 230 mg/kg BW NA intraperitoneally delivered 15 minutes prior to an intraperitoneal STZ injection at a dose of 65 mg/kg BW in 3 month old male Wistar rats. This model showed features of hyperglycemia in the non-fasted state, abnormal glucose tolerance and insulin responses as well as a 40% preservation of pancreatic insulin stores. In the latter model developed by Nakamura *et al.*, male C57BL/6J mice at 5-6 weeks of age were used and injected twice with STZ intraperitoneally at a dose of 100 mg/kg BW at day 0 and 2 of the experiment along with 240 mg/kg BW NA 15 minutes prior to each STZ injection. To this combination they also investigated the effects of a high-fat diet on this model. The high-fat-fed-240 mg/kg BW NA with 100 mg/kg BW STZ model revealed significantly elevated total cholesterol, triglycerides and HDL cholesterol compared to normal pellet diet-fed mice. The two variations also demonstrated significantly higher plasma glucose concentrations compared to normal mice. This model is advantageous as it resembles closely the diabetic pattern seen in East Asian diabetic patients, a large percentage of which are non-obese. Both models by Masiello *et al.* (1998) and Nakamura *et al.* (2006) were validated by anti-diabetic drugs and thus are suitable for drug screening and pharmacological studies, however they are only applicable for non-obese T2D.

1.6.6 Surgical rodent models of T2D

1.6.6.1 Partial pancreatectomized models

As a means to avoid liver and kidney damaged induced by alloxan, Pauls and Bancroft (1949) developed a new method to induce T2D in mice through partial pancreatectomy. In 1983, Bonner-Weir *et al.* aimed to study the consequences of reduced pancreatic β-cell mass in rats through removal of 85-90% of the pancreas. These animals displayed moderate hyperglycemia from day 4 post-surgery and was maintained for a further 6 week period. During this 6 week period, no significant differences were noted in serum insulin or body weight compared to control groups, however, after 7 weeks they had a normal fasting blood glucose and insulin concentration, and only became hyperglycaemic postprandially or after

an intraperitoneal glucose tolerance test (IPGTT). A major limitation of this model is the regeneration of the remnant pancreas, although for some researchers, studies into the adaptive mechanisms of pancreatic β-cells may be required, for which this model is particularly useful (Masiello, 2006). Srinivasan and Ramarao (2007) noted the advantages of this model being the resemblance of T2D through reduced pancreatic β-cell mass and that this model avoids any cytotoxic effect of chemical-inducers of T2D on other body organs. A more stable form of diabetes can be developed through the combination of partial pancreatectomy with chemical inducers such as STZ and alloxan. One such model, makes use of a 50% pancreatectomy in combination with 350 mg/kg BW NA prior to and after intraperitoneal injection of 200 mg/kg BW STZ in BALB/c mice. This model demonstrated stable and significantly elevated fasting blood glucose with reduced serum insulin yet a drastic loss in body weight, which is usually seen in T1D (Kurup and Bhonde, 2000). The overall disadvantage of this model is the advanced technical and surgical skills required for such studies. A well-equipped surgical laboratory is required, as this is a major surgery and the risk of infection in the animals is high. The animals must be supplied with post-surgical antibiotics as well as administration of pancreatic enzymes (Fröde and Medeiros, 2008). A potential disadvantage of this model is the development of glucose insensitivity, a feature not seen in human T2D (Masiello *et al*., 1998). Another disadvantage may be the occurrence of digestive problems due to the excision of exocrine portions of the pancreas leading to a deficiency in amylase enzymes (Srinivasan and Ramarao, 2007).

1.6.6.2 Intrauterine growth retardation models

Intrauterine growth retardation (IUGR) is the low-birth weight of infants due to the limited availability of nutrients to the fetus during gestation and is a relatively common complication of pregnancies (Simmons *et al*., 2001; Vuguin *et al*., 2004). Barker *et al*. (1993) were the first to speculate that IUGR was linked to the development of disease later in life, specifically the development of obesity, hypertension and T2D. Nutrient deficits to the fetus result in modifications to the gene expression and functionality of cells within the pancreas, liver and muscle (Peterside *et al.,* 2003). This is confirmed by earlier work by Hales *et al*. in 1991 who demonstrated that poor fetal and neonatal growth is directly linked to T2D in adulthood. IUGR causes significant reductions in pancreatic β-cell mass in neonates, which are never recovered in adulthood, resulting in impaired glucose tolerance and can lead to the development of T2D. Advantageously, these animals follow the natural pattern of disease by

developing insulin resistance as an early feature in life and later developing hyperglycemia and subsequent pancreatic β-cell dysfunction (Simmons *et al*., 2001).

In 2001, Simmons *et al.* developed an IUGR rodent model through bilateral uterine artery ligation such that blood flow to the fetus is not fully diminished but rather reflecting the human complication seen in some pregnancies. After 19 days of gestation, these rats were anesthetized with xylazine (8 mg/kg BW) and ketamine (40 mg/kg BW) intraperitoneally and both uterine arteries were cut. Neonatally these rats displayed decreases in insulin compared to controls and significantly decreased body mass. However, by week 26, IUGR rats were obese in nature, having a significantly higher fat pad mass around the body compared to the control rats. 1 week after birth, these rats displayed no significant differences in fasting blood glucose or insulin, however after week 7, there were significant differences in both fasting blood glucose and insulin compared to the control, indicating mild hyperglycemia and hyperinsulinemia. However, significantly increased insulin levels were sustained only until week 15, after which they remained non-significantly different from the control, having demonstrated a decline in insulin secretion. On the other hand, increases in fasting blood glucose seen in the IUGR group continued to rise, even after 26 weeks, having fasting blood glucose higher than 200 mg/dl, indicating a state of T2D. Studies done by Vuguin *et al.*, (2004) demonstrated that IUGR permanently alters hepatic glucose metabolism through oxidative stress mechanisms in the fetal development, thus occurring prior to the onset of obesity and hyperglycemia, thus representing an early defect, similar to insulin resistance seen in humans.

The major advantages of this model are the successful induction of insulin resistance, hyperglycemia, hyperinsulinemia, obesity and reduced pancreatic β-cell mass. However, many additional diabetic parameters including lipid profiling, liver and kidney functioning and responses to anti-diabetic agents have not yet been reported and hence remain unknown, thus compromising its suitability as a model for T2D on the whole. Moreover, the surgical ligation of uterine arteries requires advanced technical skills and the development of T2D is only seen after 3 months of age (Simmons *et al.*, 2001, Vuguin *et al.*, 2004) thus requiring a long induction period which may not be suitable for some researchers. Further investigations are required to enhance the suitability of this model, however, the literature reported thus far, indicates that it may be an appropriate model for T2D.

1.7 Statement of purpose, problem identification and aim of study

As indicated by the extensive research above, several animal models are available to date. Some include the use of large animals, such as pigs, dogs, monkeys and other primates (Srinivasan and Ramarao, 2007). These are useful for their similar size to humans and similarity to the disease phenotypes. However, the development of the diseased state in these animals requires a long time frame, making data collection difficult and husbandry expenses high (Daneshgari *et al.*, 2009). Smaller animals such as rodents are more frequently used for such research, due to their short generation time (Chen and Wang, 2005), smaller size, ease of handling due to their tranquil nature and maintenance as well as their cheaper costs (Islam and Loots, 2009).

Although models for T2D have been plentiful in the last few decades, however none are without limitations. As described above, two major types of models exist for such research: genetic models (genetically induced spontaneous models) and non-genetic models (experimentally induced non-spontaneous models). Genetic animal models include inbred strains as well as knock-out and transgenic mice (Reuter, 2007). However, there are several limitations to this approach as only 40% of patients presenting with T2D diabetes have a family history of this disorder. In addition, T2D sufferers tend to have a "polygenic mode of inheritance" as opposed to single-gene defects developed in genetic animal models of T2D (Reuter, 2007). Highly selective inbred strains also fail to account for the large heterogeneity seen in the human population suffering with T2D. Moreover, these models are expensive, not easily accessible, and tend to show features more typical of T1D as opposed to T2D (Srinivasan *et al.*, 2005). Non-genetic dietary-induced T2D models thus provide a more heterogenous approach to investigate the pathology of T2D, however most of these models do not present with closely accurate correlations to the human clinical condition (Masiello *et al*., 1998). Many of the non-genetic models as well as genetic models of T2D fail to develop heterogeneous symptoms seen in T2D patients. Non-genetic models often more closely resemble T1D, whereas genetic models exhibit single gene defects, thereby failing to demonstrate heterogeneity of symptoms seen in patients with T2D (Winzel and Ahrén, 2004). There is therefore an urgent need to develop and establish better models for T2D by either adjusting or altering existing models, developing novel methodologies or through a combination of the two (Zhang *et al.*, 2008).

The aim of this study was, therefore, to develop a novel, alternative non-genetic model, which will be inexpensive and easy to develop, and will be appropriate to generate all major pathological features of T2D. In addition, it is aimed that the model will be suitable for pharmacological screening and acute studies that can be used in developing countries that may have limited resources and funding.

CHAPTER 2 Fructose-fed-streptozotocin-injected rat model

2.1 Model development background

Animal models in diabetes research are very common where rats are the first choice of use, comprising over 85% of these models (US Department of Agriculture, 1989). Although genetic models provide a better pathogenesis for the disease, non-genetic models, are far more cost-effective, widely available and easy to develop diabetes with minimal research facilities, which is particularly useful in developing countries where funds and resources may be limited (Islam and Loots, 2009). The major disadvantages of the non-genetic type 2 diabetes (T2D) models include resembling the pathogenesis of Type 1 diabetes (T1D) more closely than the T2D. Furthermore, to date, many models have not been validated by anti-diabetic drugs thus their use and suitability for pharmacological screening is unknown (Islam and Loots, 2009). Pharmacological screening is an important aspect of T2D model development as T1D models would remain insensitive to T2D drugs. This is due to major mechanistic differences as many T2D drugs aim to increase insulin sensitivity e.g. metformin or enhance insulin secretion e.g. glibenclamide. T1D models will not respond to these drugs due to an absolute insulin deficiency arising from the autoimmune destruction of pancreatic ß-cells. Besides the disadvantage related to non-similarity of disease pathogenesis, many of the existing diet-induced models, such as fat-fed and fructose-fed models, require a long diabetes induction time, which increases the experimental cost of research (Srinivasan *et al.,* 2005, Reuter, 2007).

Successful models of T2D are required to display the two major pathogeneses of T2D: a progressive decline in insulin action (insulin resistance), followed by the inability of pancreatic β-cells to compensate for insulin resistance (pancreatic β-cell dysfunction) (Srinivasan *et al.,* 2005). The pathogenesis of diabetes in animal models is most likely similar to the pathogenesis in humans, however the dose and type of inducers and the composition of diets largely affect the success of the induction of T2D in experimental animals. Hence, many factors and situations need to be considered in order to develop an authentic animal model of T2D. Literature has shown that low fat diets are not enough to induce insulin resistance but very high fat diets in combination with a streptozotocin (STZ) injection may cause severe pancreatic β-cell damage as well as permanent insulin resistance (Reed *et al.,* 2000).

On the other hand, lower doses of STZ such as 40 mg/kg BW or less via intraperitoneal injections have been shown to be ineffective in inducing T2D whilst more than 50 mg/kg BW is usually used to induce the pathogenesis of T1D in rats (Katsumata *et al.,* 1992, Islam and Choi, 2007). Furthermore, while being used extensively in the development of diabetes, STZ is unable to induce insulin resistance directly, rather, results in hyperglycemia from direct pancreatic β-cell damage (Srinivasan *et al.,* 2005). However, the induction of insulin resistance through fructose-feeding in animals has been employed previously (Hininger-Favier e*t al.,* 2009). Fructose has been supplied *ad libitum* either in drinking water or with diets with a concentration of 10-15% for a short or longer period to induce insulin resistance or T2D respectively in experimental animals (Dai *et al.,* 1994). Unfortunately the induction of insulin resistance as well as T2D only by fructose-feeding requires several weeks which increases the cost of study. It was thus hypothesised that the combination of fructose-feeding for a shorter period of time and a lower dose of STZ injection may induce all major pathogeneses of T2D in rats.

2.2 Materials and method

2.2.1 Reagents and materials

Streptozotocin (STZ) (>98%) (Sigma-Aldrich) was purchased from Capital Lab Supplies cc. Durban, South Africa. Glucostrips, and Fructose (Nature's Choice™ Wholefood specialists, Meyerton, South Africa 1960) were purchased from a local pharmacy. Glibenclamide (Pharmacare Ltd.) and Metformin (Austell Laboratories Pvt. Ltd.) were purchased from a local pharmaceutical company (Pharmed Ltd., Durban, South Africa). A glucometer (GlucoPlus Inc, Quebec, Canada, measuring up to 600 mg/dl) was used for measuring fasting and non-fasting blood glucose levels. Serum analysis was conducted using an Automated Chemistry Analyzer (Labmax Plenno, Labtest, Lagon-Santa, Brazil) according to manufacturer's instructions.

2.2.2 Animals

Thirty-six (6 weeks old) male Sprague-Dawley rats were procured from the Biomedical Resource Unit (BRU) at Westville Campus from the University of KwaZulu-Natal, South Africa. Animals were randomly subdivided into 6 groups, each containing 6 rats each as follows: Normal Control (NC), Diabetic/Streptozotocin control (STZ), Fructose-10% (FR10+STZ), Fructose-20% (FR20+STZ), Fructose-30% (FR30+STZ) and Fructose-40% (FR40+STZ) with a mean similar weight of 190.56 g ± 23.60g. Two rats per poly-carbonated

cage were housed in a temperature and humidity controlled room with a 12 hour light-dark cycle. The rats were fed a commercially available rat chow diet *ad libitum* throughout the entire 11 week experimental period. Control groups were supplied with normal drinking water *ad libitum* whilst FR10+STZ, FR20+STZ, FR30+STZ and FR40+STZ were supplied with 10%, 20%, 30% and 40% fructose solutions respectively for 2 weeks only to induce insulin resistance. Hereafter they were supplied with normal drinking water for the remainder of the experiment. The animals were maintained according to the rules and regulations of the University of KwaZulu-Natal (UKZN) Animal Ethics Committee (Ethical approval number: 076/10/Animal).

2.2.3 Induction of diabetes

All animals received a regular chow diet whilst FR10+STZ, FR20+STZ, FR30+STZ and FR40+STZ additionally received a 10%, 20%, 30% and 40% D-Fructose respectively in drinking water for 2 weeks. Hereafter, STZ was dissolved in citrate buffer (pH 4.4). The solution was then filtered through a sterilized 0.45 millipore filter. After 2 weeks of dietary manipulation, diabetes was induced in all groups, except the NC group by a single intraperitoneal injection of STZ (40 mg/kg BW) whilst the NC group received a vehicle citrate buffer (pH 4.4) injection only.

Fasting and non-fasting blood glucose levels of all animals were measured 1 week after STZ injection. Blood was taken from rat tail veins using a 25G needle and blood glucose was measured using a portable glucometer (GlucoPlus Inc, Quebec, Canada). Animals with fasting blood glucose (FBG) level > 200 mg/dl or non-fasting blood glucose (NFBG) level > 300 mg/dl were considered diabetic. FBG levels were measured weekly throughout the experimental period until 1 week after STZ injection (Week 4). Hereafter NFBG levels were measured (Week 5-12) weekly. The rats were firstly fasted for a 12 hour period, after which food was returned *ad libitum* for 3 hours and then subsequent NFBG was measured. During the fasted period, all groups received normal drinking water and the amount consumed after the 12 hour period was recorded. During the 3 hour feeding period prior to NFBG measurements, groups consuming fructose solutions had these returned, whilst the controls groups continued to consume normal drinking water throughout this period.

2.2.3.1 Food and fluid intake, body weight and exclusion of animals

Daily food and fluid intake were monitored and weekly body weight changes were measured during the entire experimental period. Three weeks after the STZ injection, the severity of diabetes was significantly higher in the FR20+STZ, FR30+STZ and FR40+STZ groups and some of the animals from these groups were dead due to severe hyperglycemia. Therefore, the rest of the animals of these groups were excluded from the study and the remaining experiment was continued with NC, STZ and FR10+STZ groups. None of the animals were dead from these groups.

2.2.4 Oral glucose tolerance test

After an overnight fast (12 hours), rats were orally dosed with a D-glucose solution (2.0 g/kg BW) at week 6 of the experimental period. Glucose concentrations were subsequently measured by blood collection from the tail veins at 0 (just prior to oral glucose dosing), 30, 60, 90, and 120 minutes after oral dosing and blood glucose levels monitored with a glucometer.

2.2.5 Effect of anti-diabetic drugs on fructose-fed-streptozotocin-injected rats

The validity and suitability of the fructose-fed-STZ-injected rat model for pharmacological screening was determined by performing several anti-diabetic drug response tests. Two established and commonly employed, but mechanistically different, anti-diabetic drugs were used, namely insulin secretogogues such as metformin (biguanide) and glibenclamide (sulfonylurea). These tests were performed at week 7 and 9 of the experimental period respectively. Each group received a single oral dose of metformin (500 mg/kg BW) dissolved in 1% Na-CMC or a single dose of glibenclamide (5 mg/kg BW) dissolved in 1% Sodium carboxymethylcellulose (Na-CMC). For the glibenclamide test, the blood glucose concentration of each animal was measured at 0 and 180 minutes post oral administration of the drug. For the metformin test, the blood glucose concentration of each animal was measured at 0, 30, 60, 120 and 180 minutes post oral ingestion of the drug. A one week drug wash out period (week 8) was required between anti-diabetic drug response tests to ensure no interference of drugs between testing.

2.2.6 Collection of blood, liver, pancreas, kidneys and heart

At the end of the experimental period animals were fasted for 14 hours prior to euthanasia by halothane anaesthesia. Animals were weighed before euthanasia after which blood, liver, heart, kidneys and pancreas were collected. Blood was collected through cardiac puncture using a 21G needle and fasting blood glucose levels were immediately measured using a portable glucometer. Blood samples were subsequently placed in heparin tubes and allowed to coagulate on ice for approximately 3 hours and then centrifuged at 3000rpm for 15 minutes. Hereafter, serum was separated into labelled microtubes and stored at -30˚C for further analysis. Liver, kidney and heart samples were washed in cold 0.9% saline solution, wiped dry with filter paper, weighed on an analytical balance and preserved at -30˚C for further analysis. Part of the pancreas sample was immediately fixed in 10% neutral buffered formalin solution for histopathological examinations, which was replaced weekly during the entire preservation period. The remainder of the pancreas was preserved at -30˚C for further analysis.

2.2.7 Analytical methods

Liver glycogen concentration was measured photometrically by using the phenol-sulphuric acid method as described by Lo *et al*. (1970). Serum insulin was analyzed using an ultra sensitive rat insulin ELISA kit (Mercodia AB, Uppsala, Sweden) using a multi plate ELISA reader (Biorad-680, BIORAD Ltd., Japan). HOMA-IR and HOMA-beta scores were calculated using fasting serum insulin and FBG concentrations at the end of the experimental period according to the following formula:

HOMA-IR = [Insulin (U/L) x Blood glucose (mmol/L)]/ 22.5

HOMA-beta = [20 x Insulin (U/L)] / [Blood glucose (mmol/L) – 3.5]

Conversion factor: Insulin (1U/L = 7.174pMol/L) and Blood glucose (1mmol/L = 18 mg/dl)

Serum lipid profiles, serum creatinine and liver function enzymes (AST and ALT) were measured using an automated chemistry analyzer (Labmax Plenno, Labtest, Brazil). Total cholesterol was measured directly in the serum according to the following principle:

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{Cholest-4-en-one} + H_2O_2.$$

$$H_2O_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{Quinonemine} + 4H_2O.$$

Quinonemine is maximally absorbed at 500 nm. The intense red colour generated is directly proportional to the cholesterol concentration in the sample. LDL cholesterol was quantitatively precipitated after centrifugation. Triglycerides are measured in a similar manner to total cholesterol to yield quinonemine. The red colour that is produced is directly proportional to triglyceride concentration.

Creatinine was measured according to the following test principle:

$$\text{Creatinine} + \text{alkaline picrate} \; - - - \rightarrow \text{Creatinine picrate}$$

This yields an intense red colour. This red colour is directly proportional to the concentration of creatinine at 510 nm.

Liver enzyme ALT was measured according to the following test principle:

$$\text{L-Alanine} + \text{ketoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L-glutamate}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}$$

NADH oxidation at 340 nm is directly proportional to the ALT activity in a sample. AST is measured in an almost identical manner:

$$\text{L-Aspartate} + \text{ketoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L-glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}$$

2.2.8 Liver glycogen content

Liver glycogen content was measured photometrically according to the Phenol-Sulphuric acid Method developed by Lo *et al.*, (1970). Liver samples of ≤ 1.0g were measured and placed at the bottom of a screw-capped pyrex tube using forceps and then placed on ice. The sample was then immersed in 1.5ml of 30% KOH saturated with Na_2SO_4. This was then placed in a boiling water bath for 20-30 minutes. The sample was then removed and allowed to cool on ice. 2ml of 95% ethanol was then added and placed on ice for a minimum of 30 minutes. Samples were centrifuged at 840 x g (2812 rpm) for 25-30 minutes. The supernatant was aspirated and the glycogen precipitate was dissolved in 3.0ml distilled water. 50µl of this solution was transferred to a clean test tube containing 450µl distilled water. This was performed in duplicate. Glycogen standards were prepared from a stock solution of 1 mg/ml

glycogen (Oyster, Type II, SIMGA-ALDRICH, USA) at concentrations of 10, 20, 40, 80, 160, 320 and 640µg/ml. To both the standards and samples, 0.5ml of 5% phenol was added to each test tube, followed by the rapid addition of 2.5ml 96-98% H_2 SO_4. This was left to stand at room temperature for 10 minutes, after which the absorbance was read in a spectrophotometer at 490nm. A solution of distilled water served as the blank. The concentration of liver glycogen was thereafter calculated from a liver glycogen standard curve. This experiment was performed in duplicate.

2.2.9 Serum insulin analysis

Rat serum analysis was performed using a Ultrasensitive Rat Insulin ELISA kit from Mercodia (Uppsala, Sweden). This kit provides a method for the quantitative determination of serum insulin. It makes use of a direct technique whereby 2 monoclonal antibodies act against different antigenic sites on the insulin molecule. Insulin contained in the rat serum reacts with a peroxidase-conjugated anti-insulin antibody during incubation. The several washing steps remove any unbound enzyme-labelled antibodies. The bound conjugate is then detected through the reaction with 3,3',5,5'-tetramethylbenzidine. This reaction is then stopped by the addition of sulphuric acid giving rise to a yellow colour that is read photometrically at 450nm. This experiment was performed through the development of a serum insulin standard curve using 50µl of a range of calibrators from Calibrator 0-7. In separate wells, 50µl of serum sample was added after which 50µl of enzyme conjugate was added to all wells. This was incubated on a shaker at 700-900rpm for 2 hours at room temperature. Hereafter, the solution was aspirated followed by 6 wash steps of 350µl of 21x wash buffer. At the last wash step, the plate was inverted and tapped firmly against absorbent paper towel. 200µl of Substrate TMB was added to all wells. This was incubated for 30 minutes in which time a blue solution developed. After 30 minutes, a stop solution comprised of acid was added to all wells in 50µl aliquots. This was shaken for 5 seconds to ensure adequate mixing of Substrate TMB and the stop solution. The absorbance was read in triplicate using a multiplate reader (Biorad-680, BIORAD Ltd., Japan) at 450nm.

2.2.10 Histopathological examination of pancreatic islets and staining

Immediately after pancreatic organ collection, a small section of the pancreas was cut and placed in a 1ml eppendorf tube containing 10% neutral buffered formalin. The formalin solution was changed weekly until tissue processing. For tissue processing, the section of pancreas was dehydrated in 2 changes of 70% ethanol for 1 hour each, followed by 1 change

in 80% ethanol for 1 hour, 1 change in 90% ethanol for 1 hour and 1 overnight change in 100% ethanol. The following day, the tissues were cleared in 3 changes of xylene for 1.5 hours each. The tissue sections were embedded in paraffin wax. The paraffin blocks were trimmed to an appropriate size. Sections were cut at 3µm and placed in a water bath at 40-45ºC. The sections were mounted onto slides and allowed to air-dry overnight. The following day, the slides containing the cut sections were deparaffinized in 2 changes of xylene for 5 and 2 minutes respectively. The slides were rehydrated in 1 change of 100% ethanol for 2 minutes, 1 change of 90% ethanol for 2 minutes, 1 change of 70% ethanol for 2 minutes, 1 change of 50% ethanol for 2 minutes and placed in distilled water. The slides were stained for 5 minutes using hematoxylin as the primary stain, and washed in running tap water. Hereafter the slides were stained for 3 minutes in eosin as a counterstain. The slides were then dipped in 90-100% ethanol followed by xylene, wiped and coverslipped in DPX mounting agent. The slides were then viewed using a light microscope (Olympus CKX41, Olympus, Japan) connected to a computer.

2.2.11 Statistical analysis

All data are presented as mean ± SD. The data were analyzed by a statistical software package (Statview, Version 5.0, Cary, NC, USA) using the Tukey-Kramer multiple range *post-hoc* test and Tukey-Kramer paired T-test. The values were considered significantly different at $p < 0.05$.

2.3 Results

2.3.1 Daily food intake

Figure 12 shows the mean food intake (g) per animal per day over the 11 week experimental period. Food intake was measured daily at 9 am. FR10+STZ group displayed non-significantly higher food intake compared to STZ and NC, and similar results were observed for STZ compared to NC.

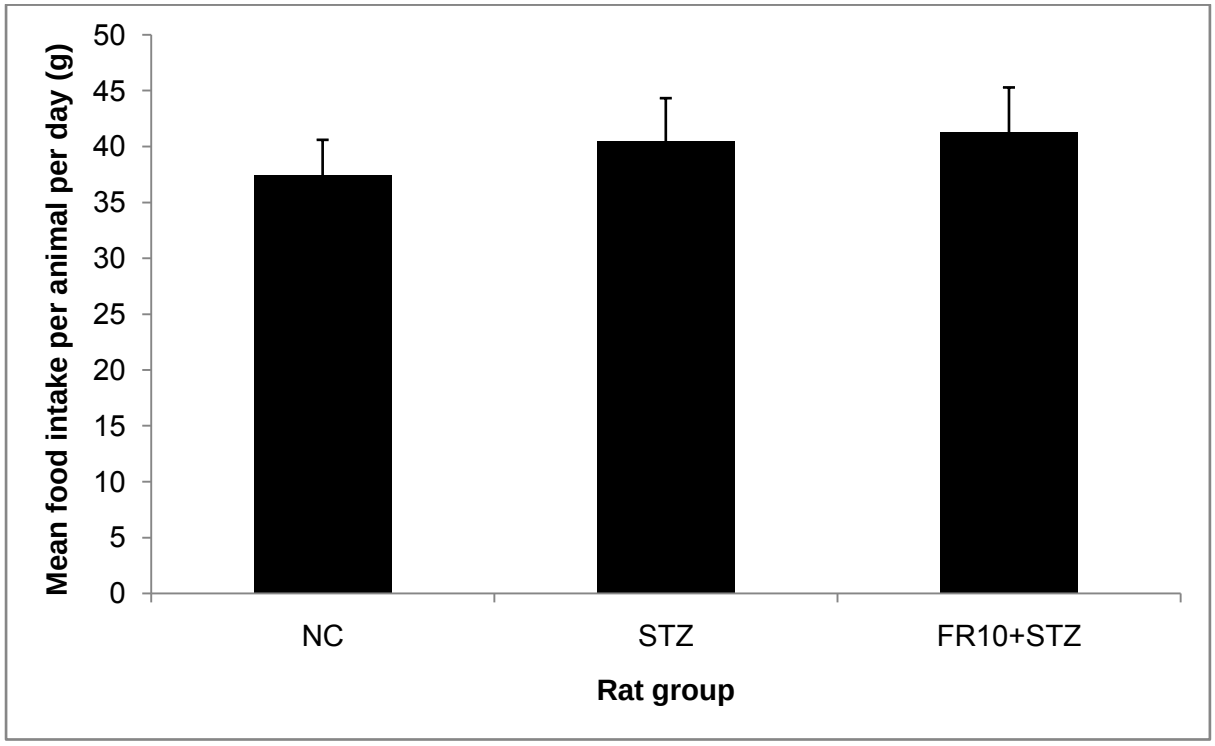

Figure 12: Mean food intake (g) per animal per day over the 11 week experimental period. Data are expressed as mean ± SD of 6 animals, where NC: Normal Control, STZ: Streptozotocin Control, FR10+STZ: Fructose 10%+STZ.

2.3.2 Daily fluid intake

Figure 13 shows the mean fluid intake (ml) per animal per day. The FR10+STZ group reveals significantly higher ($p < 0.05$) fluid intake compared to NC and STZ, whereas no significant differences were noted between NC and STZ groups. FR10+STZ group was the only group to receive 10% fructose solution in drinking water a*d libitum* for the initial 2 weeks, and hereafter received normal drinking water *ad libitum* for the remainder of the experiment. NC and STZ received normal drinking water *ad libitum* throughout the entire experimental period.

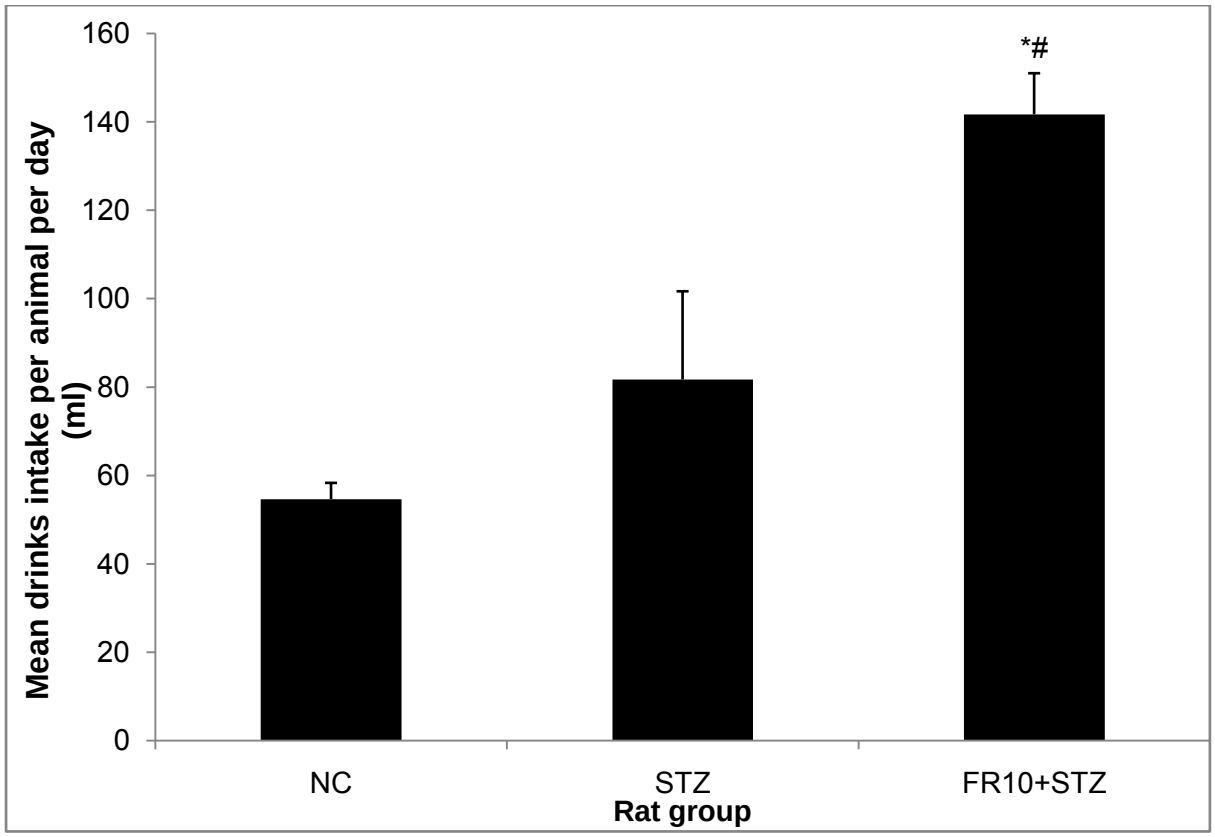

Figure 13: Mean fluid intake (ml) per animal per day over the 11 week experimental period. Data are expressed as mean ± SD of 6 animals, where NC: Normal Control, STZ: Diabetic Control, FR10+STZ: Fructose 10%+STZ. *$p < 0.05$ vs. NC and #$p < 0.05$ vs. STZ (Tukey-Kramer multiple range *post-hoc* test).

2.3.3 Weekly body weight (BW) gain

Figure 14 shows the weekly BW gain over the 11 week experimental period. During the first 3 weeks, no significant differences were noted in BW gain between the animal groups. After the injection of STZ, the BW gain of the FR10+STZ group was significantly reduced compared to the NC group. Minor weight loss was seen in the STZ and FR10+STZ groups one week after the STZ injection, however this is a common effect of STZ and is recovered quickly. Weeks 4-11 revealed steady weight gain amongst all animal groups with minor weight loss in week 11 due to the fast incurred prior to euthanasia by halothane anaesthesia.

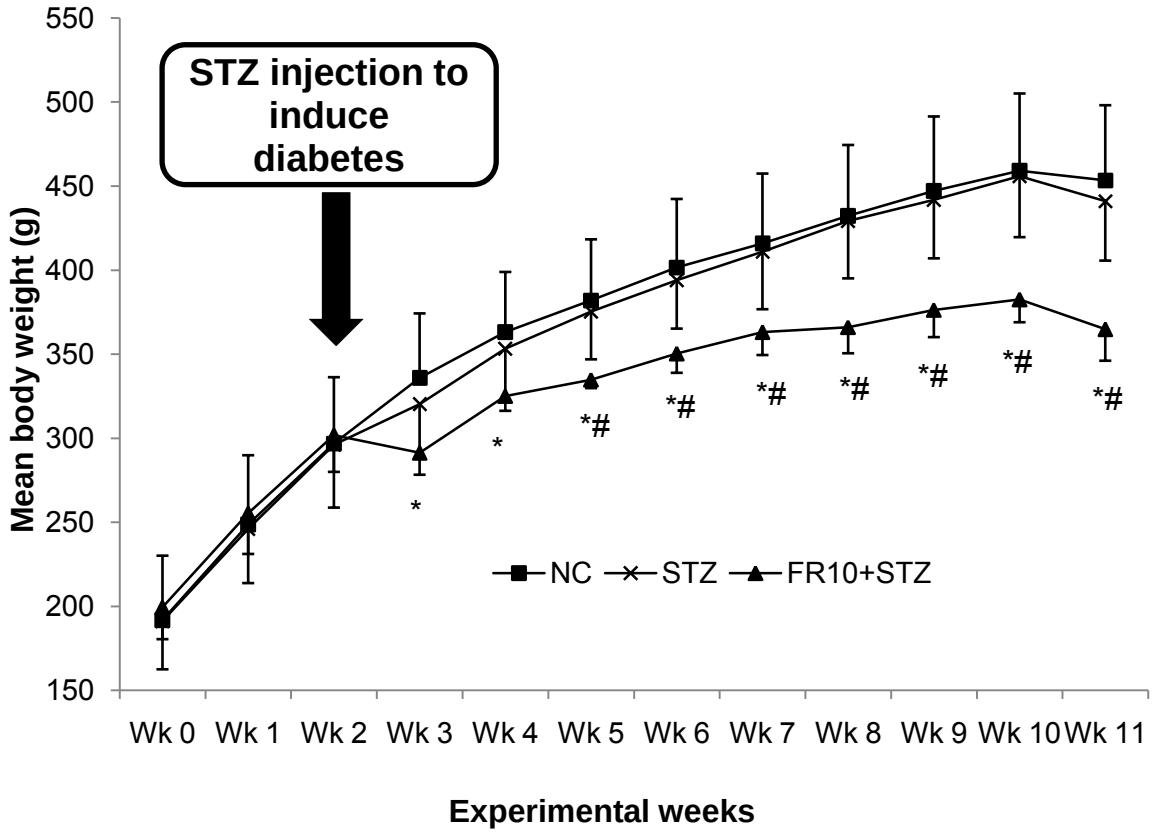

Figure 14: Mean weekly BW gain over the 11 week experimental period. Data are expressed as mean ± SD of 6 animals. *p < 0.05 vs. NC and #p < 0.05 vs. STZ (Tukey-Kramer multiple range *post-hoc* test).

2.3.4 Weekly blood glucose

Figure 15 shows the mean weekly blood glucose levels (mg/dl) throughout the experimental period. FBG revealed no significant differences between the animal groups, including the week after STZ injection. NFBG on the other hand revealed significantly higher blood glucose in the FR10+STZ group compared to both the STZ and NC groups. The STZ group also demonstrated significantly higher NFBG compared to the NC group. The STZ group did not maintain a NFBG > 300 mg/dl throughout the experimental period, however, the FR10+STZ group maintained NFBG between 350-400 mg/dl for the duration of the experiment. NFBG > 300 mg/dl was considered as a scale for type 2 diabetes.

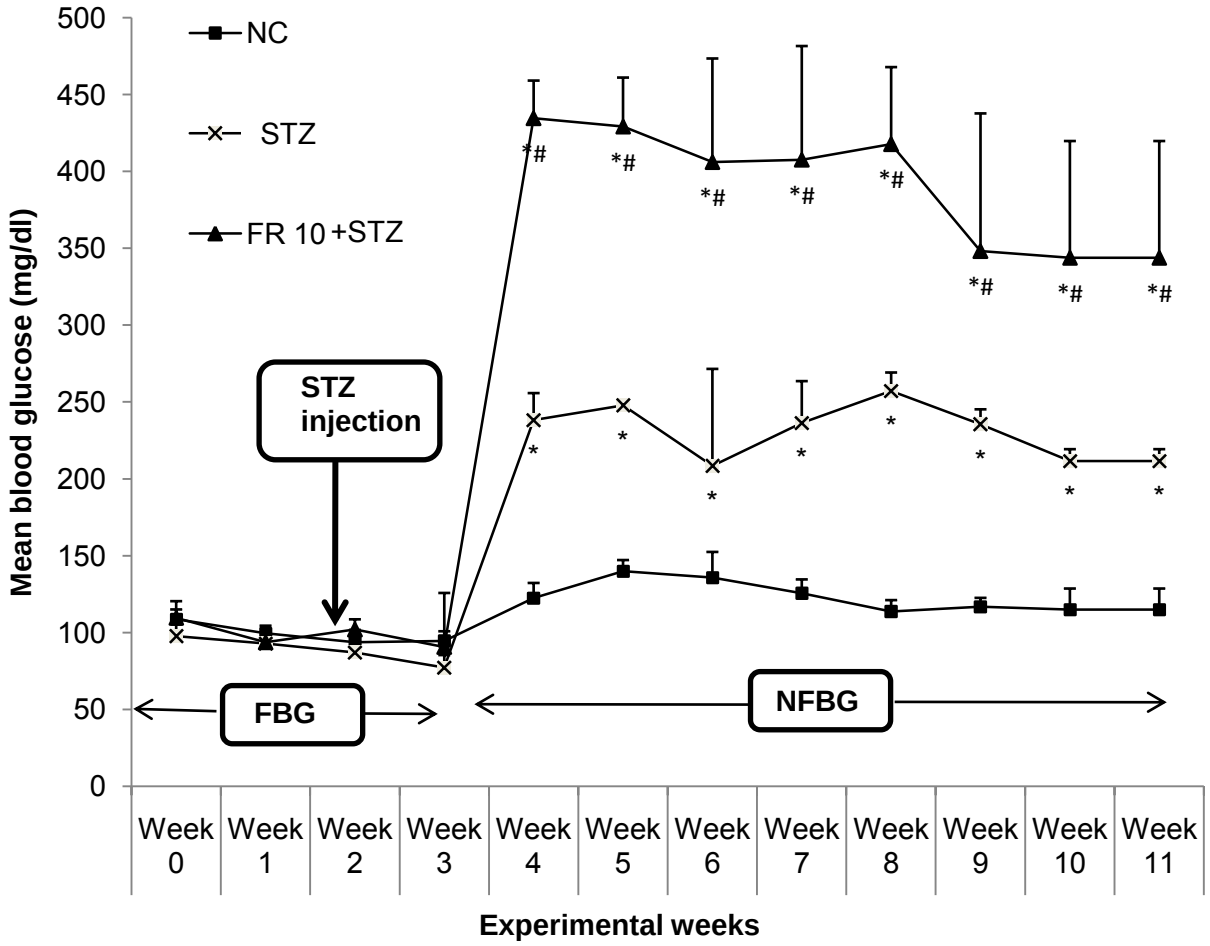

Figure 15: Mean blood glucose (mg/dl) over the 11 week experimental period. Week 0-3 are indicative of fasting blood glucose (FBG), whilst week 4-11 are indicative of non-fasting blood glucose (NFBG). Data are expressed as mean ± SD of 6 animals. *p < 0.05 vs. NC and #p < 0.05 vs. STZ (Tukey-Kramer multiple range *post-hoc* test).

2.3.5 Oral glucose tolerance test (OGTT)

The data for OGTT are displayed below in Figure 16. OGTT is a clinical diagnostic test to assess glucose tolerance ability of the body over 2 hours and can determine glucose intolerance as well as T2D. All animals were fasted overnight prior to oral dosing after which and a 2g/kg BW solution of D-glucose was administered to all rats. The blood glucose of all animals was measured at 30 minute time intervals over a 2 hour period. The blood glucose of the STZ and FR10+STZ groups were significantly higher than the NC group. The STZ group demonstrated a sharp decline in blood glucose after 90 minutes, with a blood glucose level below 200 mg/dl after 2 hours. The blood glucose of the FR10+STZ group remained elevated over 300 mg/dl almost for the entire 2 hour period.

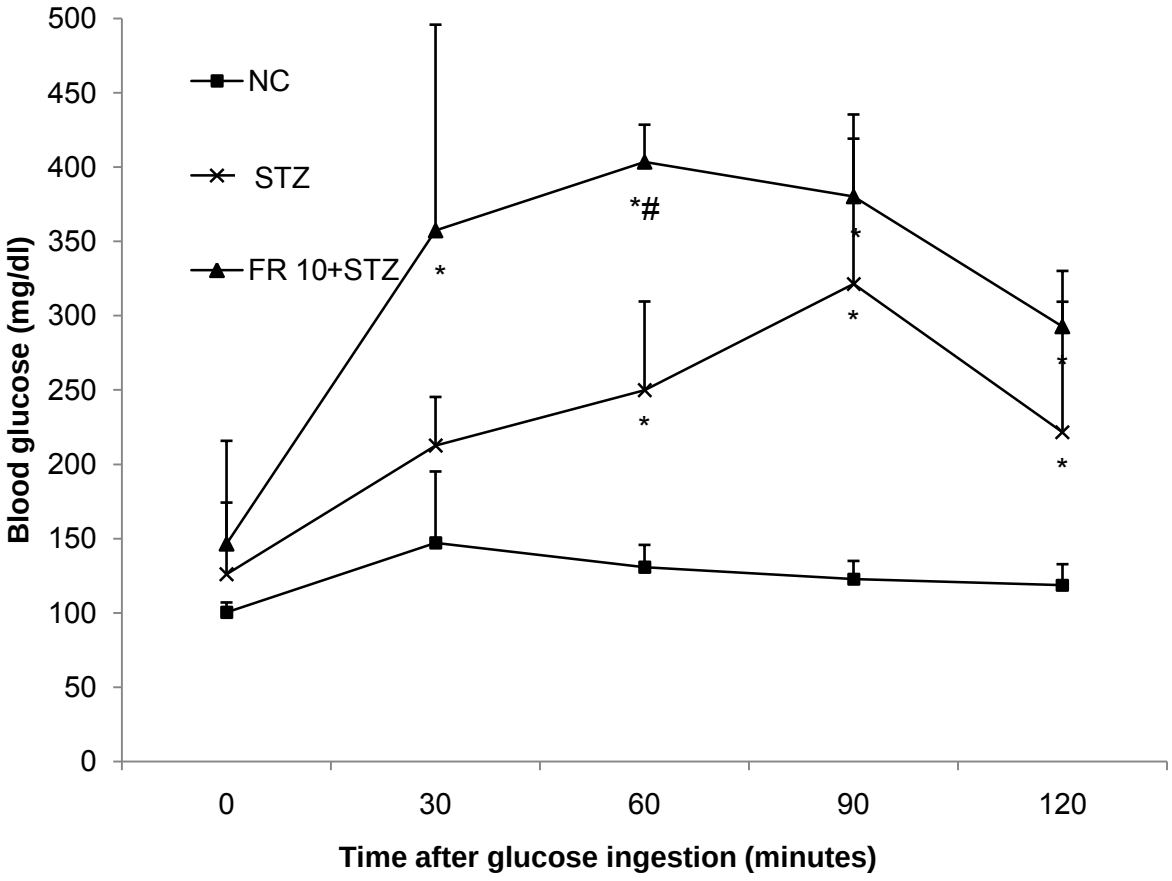

Figure 16: Oral Glucose Tolerance Test (OGTT) over 2 hours at week 6 of the 11 week experimental period. Data are expressed as mean ± SD of 6 animals. *$p < 0.05$ vs. NC and #$p < 0.05$ vs. STZ (Tukey-Kramer multiple range *post-hoc* test).

2.3.6 Effect of anti-diabetic drugs on the FR10+STZ group

In order to assess and validate the model's potential and suitability for routine pharmacological screening, Figure 17 and 18 showed the effect of two commonly used anti-diabetic drugs on the FR10+STZ group. In the case of both tests, blood samples from the FR10+STZ group were collected from tail veins just prior to drug administration (0 minutes), and after 180 minutes. When dosed with metformin, the blood samples were additionally collected at 30, 60, 120 and 180 minutes after drug ingestion. In both cases, blood glucose was significantly reduced after 180 minutes, and in the case of metformin, significant reductions were observed at both 120 and 180 minutes compared to 0 minutes. Glibenclamide significantly reduced blood glucose from 539.2 ± 58.20 mg/dl to 463.9 ± 32.48 mg/dl, whereas metformin significantly reduced blood glucose from 467.2 ± 92.72 mg/dl to 272.8 ± 67.56 mg/dl in a 3 hour period.

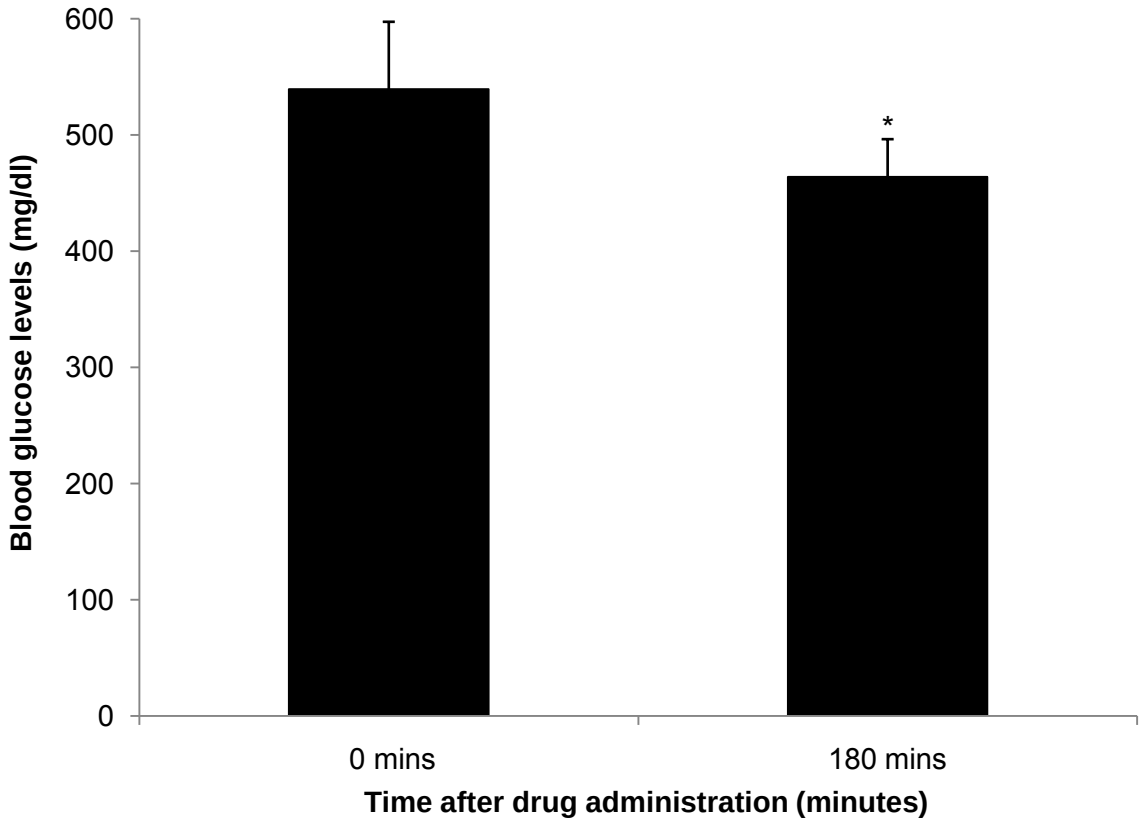

Figure 17: Effect of glibenclamide (5 mg/kg BW) on blood glucose of FR10+STZ group over a 3 hour period at week 7 of the experimental period. Data are expressed as mean ± SD of 6 animals. *$p < 0.05$ vs. 0 minutes (Student T-test).

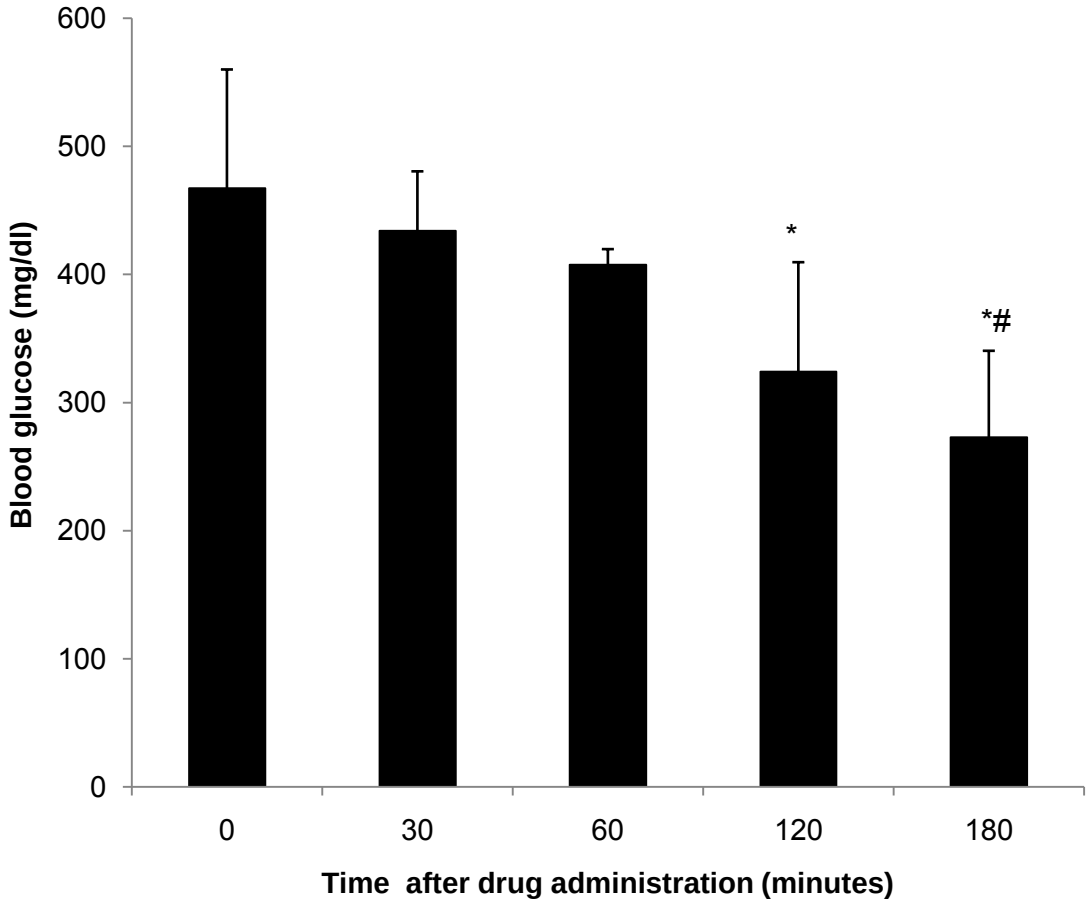

Figure 18: Effect of metformin (500 mg/kg BW) on blood glucose (mg/dl) over a 3 hour period in FR10+STZ group at week 9 of the experimental period. Data are expressed as mean ± SD of 6 animals. *p < 0.05 vs. 0 minutes and #p < 0.05 vs. 120 minutes (Tukey-Kramer multiple range *post-hoc* test).

2.3.7 Serum lipid profile

Table 2 shows the serum lipid profile of the different animal groups. FR10+STZ displayed significantly higher total cholesterol and serum triglyceride levels compared to the NC group, however, no significant differences for HDL or LDL cholesterol were observed between the groups.

Table 2: Serum lipid profile of different animal groups at the end of the experimental period.

Rat groups/ serum lipids	Normal Control (NC)	Diabetic Control (STZ)	Fructose-10% (FR10+STZ)
	mg/dl		
Total cholesterol	69.40 ± 9.26	80.80 ± 13.48	90.50 ± 14.01*
HDL cholesterol	18.60 ± 3.91	26.00 ± 8.09	24.83 ± 3.76
LDL cholesterol	32.12 ± 11.01	33.40 ± 13.13	39.10 ± 12.43
Triglycerides	93.40 ± 21.55	107.00 ± 17.38	132.83 ± 18.59*

Data are shown as mean ± SD of 6 animals.*$p < 0.05$ vs. NC. (Tukey-Kramer multiple range *post-hoc* test).

2.3.8 Liver weights and liver glycogen

Table 3 shows a non-significantly increased liver weight and relative liver weight % ([liver weight/body weight] x 100 %) in the FR10+STZ group compared to the control groups. Significant increases in liver glycogen were, however, noted in the diabetic (STZ and FR10+STZ) groups compared to the NC group.

Table 3: Liver weights and liver glycogen levels in different animal groups at the end of the experimental period.

Rat groups	Normal Control (NC)	Diabetic Control (STZ)	Fructose-10% (FR10+STZ)
Liver weight (g)	14.81 ± 2.65	13.71 ± 1.26	15.30 ± 0.99
Relative liver weight (%)	3.13 ± 0.23	4.10 ± 0.08	4.22 ± 0.22
Liver glycogen (mg/g tissue)	2.66 ± 1.23	22.05 ± 1.55*	23.81 ± 0.66*

Data are shown as mean ± SD of 6 animals.

*$p < 0.05$ vs. NC (Tukey-Kramer multiple range *post-hoc* test).

2.3.9 Liver function enzymes and serum creatinine

Table 4 shows the levels of liver function enzymes and serum creatinine in the serum of different animal groups. The highest AST concentration amongst the groups was observed in the FR10+STZ group; however the differences were not significant. The FR10+STZ group displayed a significantly higher concentration of ALT compared to the NC group, however, no significant differences were noted between the NC and STZ groups. Serum creatinine, on the other hand, can be used to assess insulin resistance indirectly. The STZ and FR10+STZ groups displayed significantly lower serum creatinine compared to the NC group at the end of the experimental period.

Table 4: Serum AST, ALT and creatinine levels at the end of the experimental period.

Rat group	Normal Control (NC)	Diabetic Control (STZ)	Fructose-10% (FR10+STZ)
AST (U/L)	91.00 ± 25.56	89.40 ± 12.12	95.50 ± 16.92
ALT (U/L)	47.20 ± 3.03	58.40 ± 12.10	66.83 ± 15.78*
Creatinine (mmol/L)	0.48 ± 0.06	0.38 ± 0.07*	0.33 ± 0.05*

Data are shown as mean ± SD of 6 animals. *p < 0.05 vs. NC (Tukey-Kramer multiple range *post-hoc* test). AST: aspartate transaminase, ALT: alanine transaminase

2.3.10 Serum insulin and HOMA-IR and HOMA-beta scores

Analysis of serum insulin concentration seen in Table 5 demonstrated a significantly lower serum insulin concentration in FR10+STZ compared to the control groups, however there was no significant difference between STZ and NC. HOMA-IR (insulin resistance) and HOMA-beta (pancreatic ß-cell function) are independent predictors of T2D. They are calculated based on fasting blood glucose and fasting serum insulin concentrations. From the calculation given below, Table 6 shows the HOMA-IR (insulin resistance) score was significantly higher in the FR10+STZ group compared to the controls and the HOMA-beta (pancreatic ß-cell function) score was significantly lower in the STZ and FR10+STZ groups compared to the NC group.

HOMA-IR = [Insulin (U/L) x Blood glucose (mmol/L)]/ 22.5

HOMA-beta = [20 x Insulin (U/L)] / [Blood glucose (mmol/L) – 3.5]

Conversion factor: Insulin (1U/L = 7.174pMol/L) and Blood glucose (1mmol/L = 18 mg/dl)

Table 5: Serum insulin and calculated Homeostasis Model Assessment (HOMA) scores at the end of the experimental period.

Rat groups	Normal Control (NC)	Diabetic Control (STZ)	Fructose-10% (FR10+STZ)
Insulin (pmol/L)	121.79 ± 43.70	79.13 ± 22.38	65.45 ± 20.77$^{*\#}$
HOMA-IR	4.35 ± 1.12	4.55 ± 2.69$^{\#}$	9.44 ± 2.35*
HOMA-B	104.94 ± 40.83	40.78 ± 16.19$^{\#}$	29.37 ± 6.75*

Data are shown as mean ± SD of 6 animals. The abbreviations denote HOMA-IR: Homeostasis model assessment of insulin resistance, HOMA-B: Homeostasis model assessment of pancreatic ß-cell function. *p < 0.05 vs. NC and #p < 0.05 vs. STZ (Tukey-Kramer multiple range *post-hoc* test).

2.3.11 Histopathological examination of pancreatic tissue

The slides for histopathological examination of pancreatic tissues are presented in Figures 19-21. The slides revealed a reduced pancreatic ß-cell numbers in the STZ and FR10+STZ groups compared to the NC group. Severe damage of pancreatic islets was seen in the STZ group with deformed ß-cells. On the other hand, FR10+STZ group revealed a significantly smaller islet size, yet better physiological shape with a considerable number of healthy β-cells compared to STZ group.

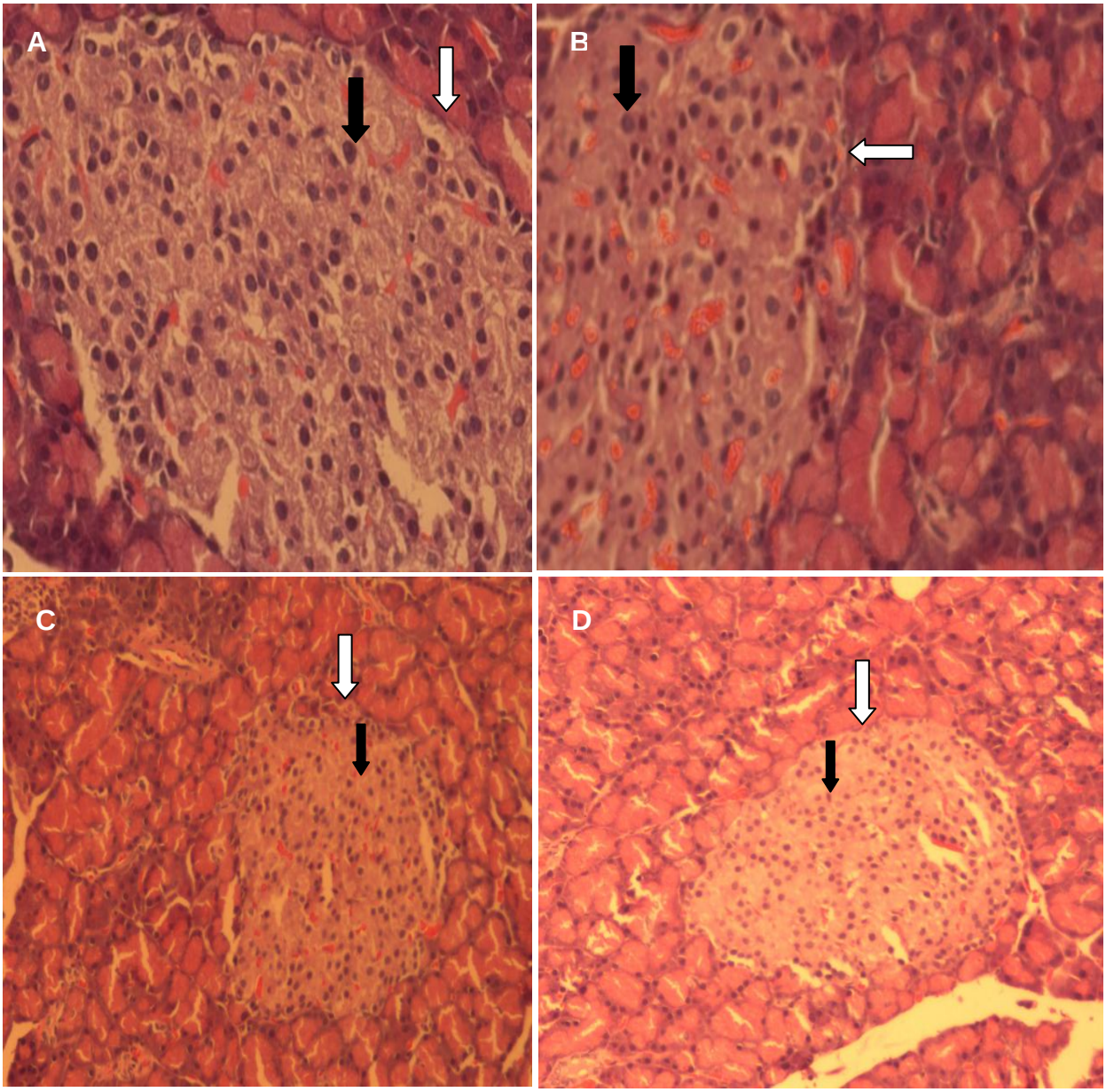

Figure 19a-d: Pancreatic islets containing β-cells in the NC group (a & b x1000; c & d x400). White arrows indicate pancreatic islets, with dark round spots inside (black arrows) indicating pancreatic β-cell.

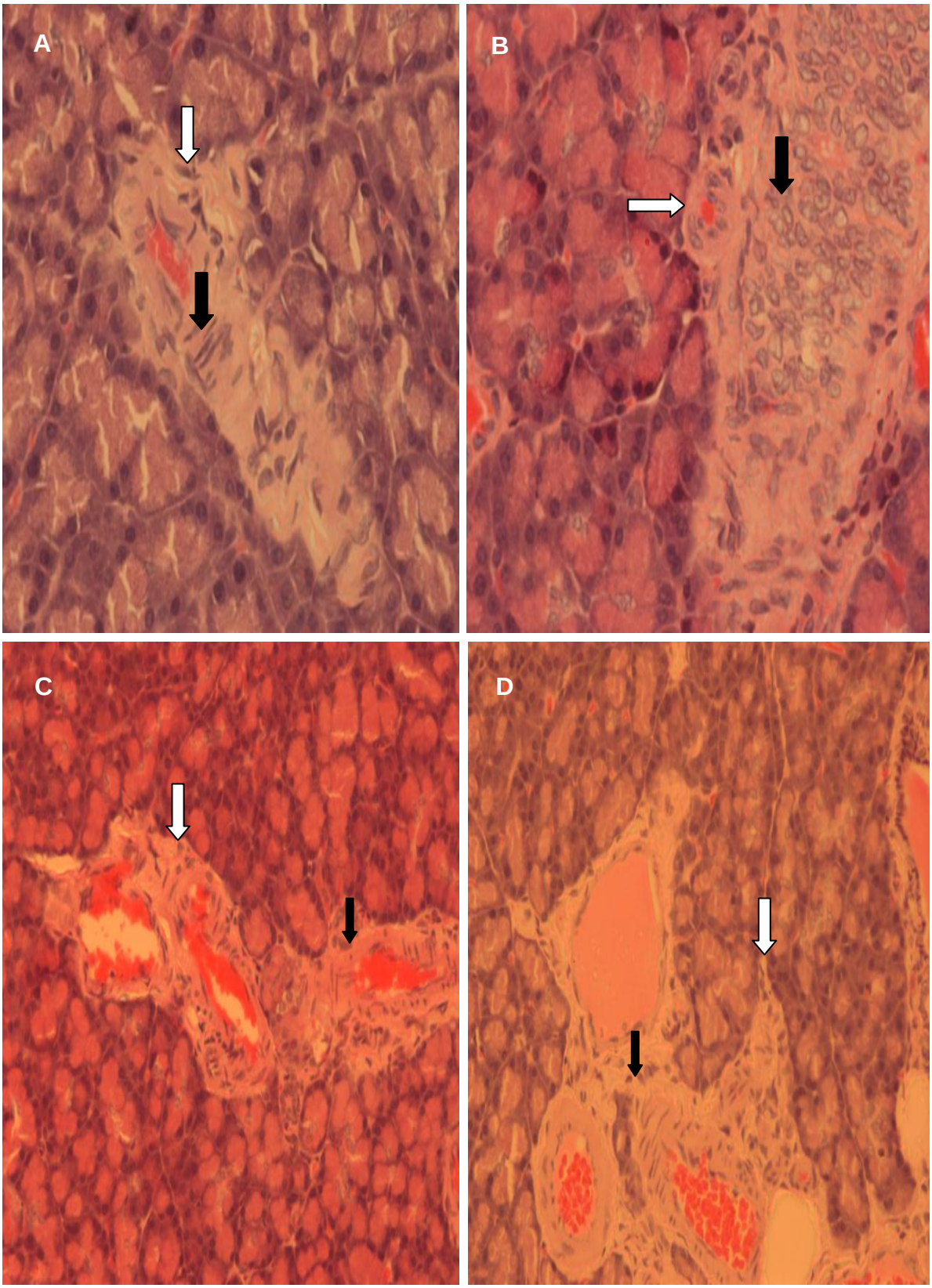

Figure 20a-d: Pancreatic islets containing β-cells in the STZ group (a & b x1000; c & d x400). White arrows indicate pancreatic islets, with dark mass inside (black arrows) indicating pancreatic β-cell.

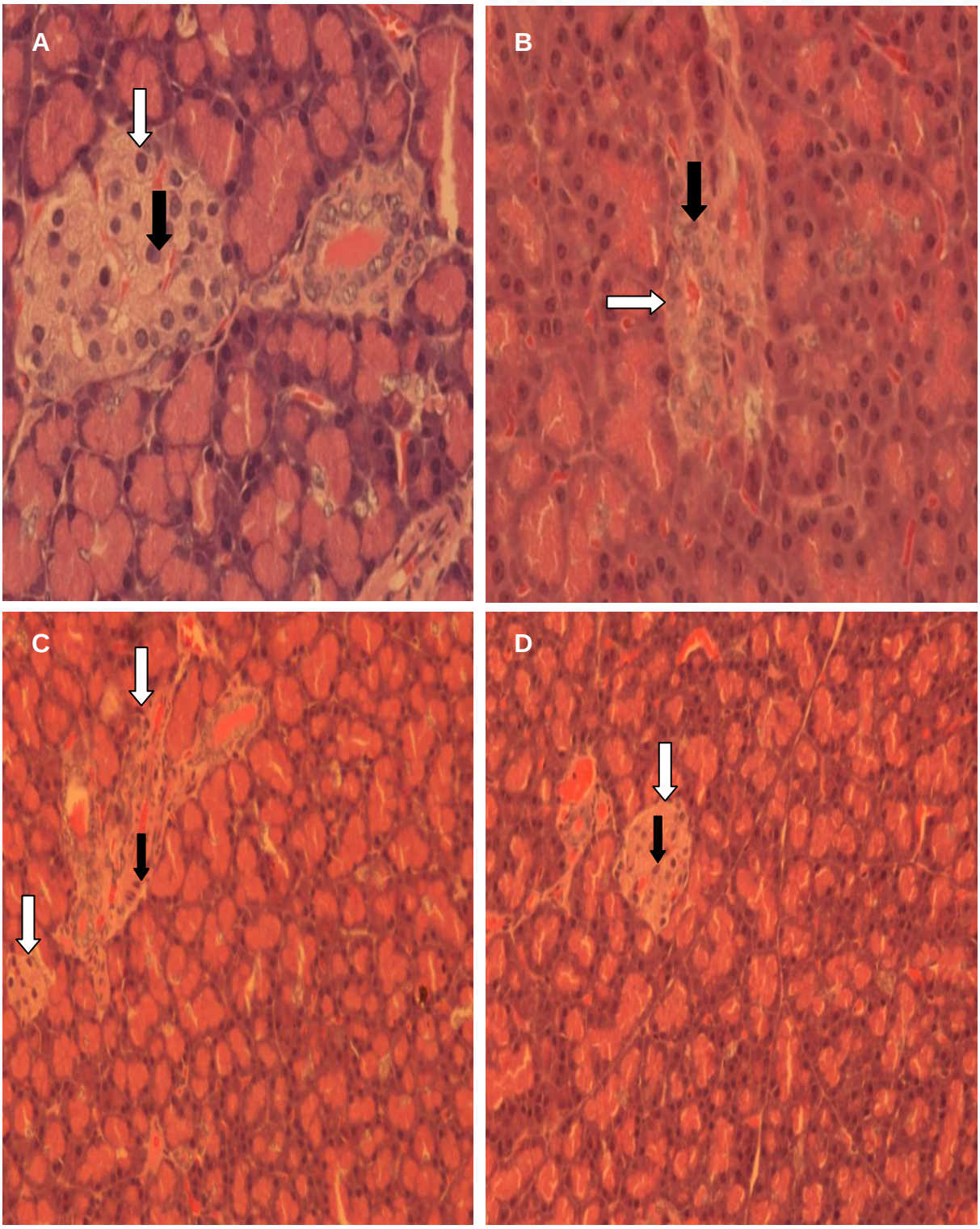

Figure 21a-d: Pancreatic islets containing β-cells in the FR10+STZ group (a & b x1000; c & d x400). White arrows indicate pancreatic islets, with dark round spots inside (black arrows) indicating pancreatic β-cell.

2.4 Discussion

The primary objective of the study was to develop an alternative non-genetic rat model for T2D that may mimic the clinical pathogeneses seen in humans particularly insulin resistance and partial pancreatic β-cell dysfunction and which can be developed in an easier way and within a shorter period of time compared to several existing non-genetic animal models of T2D (Masiello *et al.,* 1998, Reed *et al.,* 2000, Srinivasan *et al.,* 2005). Our results demonstrate that feeding a 10% fructose solution in combination with a 40 mg/kg BW STZ injection can be an easier and quicker way for the development of T2D in rats.

According to the American Diabetes Association (ADA), the most common symptoms of diabetes include polydipsia and weight loss, which is sometimes accompanied by polyphagia and blurred vision (ADA, 2007). The polydipsia but not polyphagia was evidently present in the diabetic groups in our experiment. The significantly higher fluid intake but significantly lower BW gain in the FR10+STZ group compared to the control groups might be due to the severity of diabetic condition as well as higher energy expenditure via urinary glucose excretion in our experiment. In contrast to fat-fed-STZ-injected rat models, our model did not show a higher mean BW gain compared to NC rats. The fat-fed-STZ-injected rat model attributed the higher weight gain due to the intake of high fat diet which being deposited as fat pads around the body (Srinivasan *et al.,* 2005). In our experiment, the FR10+STZ group did not exhibit actual weight loss as seen in T1D patients and animal models (Hessner *et al.,* 2004) but rather decreased weight gain as compared to the control groups.

The ADA also reported that the FBG >100 mg/dl but <126 mg/dl, blood glucose at 2 hours post-glucose load >140 mg/dl but <200 mg/dl and fasting plasma glucose (FPG) >140 mg/dl and/or 2 hours post-glucose load >200 mg/dl are respectively considered as impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and diabetes (ADA, 2007). On the other hand, recently it has been reported that FBG cannot be always considered as an authentic marker for the diagnosis of T2D (Islam, 2011). In our experiment, FBG levels in the first two weeks and even one week after the STZ injection were not significantly different between the groups and they were at normal glucose level (<100 mg/dl mean). Hence, NFBG was measured during the remaining period of the experiment. The NFBG of STZ and FR10+STZ groups were significantly higher than the NC group (Fig. 3). However, the NFBG range of FR10+STZ group was significantly higher than the STZ group (300-450 vs. 200-250 mg/dl). Like many other studies (Can *et al.,* 2011, Islam and Choi, 2008, Srinivasan *et al.,* 2005) the

NFBG >300 mg/dl has been used as a cut-off point for T2D which has been maintained by FR10+STZ but not by STZ group in our experiment. Additionally, our model demonstrated sensitivity to both metformin (glucophage) and glibenclamide by significantly reducing blood glucose levels over a 3 hour period.

Not always but in many cases hyperinsulinemia, besides hyperglycemia, has been reported as a consequence of T2D. As the disease state progresses to chronic hyperglycemia in Type 2 diabetics, insulin secretions become impaired and decline (Nattrass and Bailey, 1999). It has been reported that dietary fructose stimulates less insulin secretion but increased food intake compared to glucose and glucose-containing carbohydrates in humans (Bantle and Slama, 2006). Our results demonstrated a significantly ($p < 0.05$) lower serum insulin concentration in FR10+STZ rats as compared to NC rats (Table 5). FR10+STZ rats also displayed a lower serum insulin concentration than STZ rats indicating that fructose may play a role in the reduction of circulating insulin possibly due to its very high glycation potentials (Obrosova, 2005) that could lead to the formation of advance glycation end products with concomitant generation of oxidative stress. This oxidative stress has been reported as a major contributor to the β-cell damage in T2D (Obrosova, 2005), thus, partial pancreatic ß-cell dysfunctioning might not be solely attributed to STZ. Based on our histopathological findings, however, we speculate that the combined effect of STZ and fructose in fact minimizes the oxidative damage on pancreatic β-cells than the oxidative stress that would be produced by the individual agents alone.

In addition to serum insulin concentrations, HOMA-beta scores are often used to validate pancreatic β-cell function (Matthews *et al.,* 1985, Song *et al.,* 2007). Our results show a dramatic decrease ($p < 0.05$) in HOMA-beta scores in FR10+STZ groups as compared to NC rats. Although the use of STZ amongst researchers is very common for the induction of diabetes or hyperglycemia (Etuk, 2010, Koulmanda *et al.,* 2003), higher doses of STZ induced models resembling T1D. Hence the development of insulin resistance along with pancreatic β-cell dysfunction by using a single diabetic inducer may not be possible. Several attempts have been made to combat this shortcoming to induce typical pathogenesis of T2D, through the use of high-fat diet-fed STZ-injection (Reed *et al.,* 2000, Srinivasan *et al.,* 2005), multiple low-dose STZ injections (Zhang *et al.,* 2008) and a combination of nicotinamide and STZ injection (Masiello *et al.,* 1998, Nakamura *et al.,* 2006) in several previous studies. However, most of these models did not display the evidence of actual pancreatic β-cell damage.

In order to understand the level of pancreatic damage in our model, histopathological examinations of pancreatic islets were performed. Although the pancreatic weights were not significantly reduced (data not shown), the pancreatic islet size in FR10+STZ group were significantly reduced compared to the STZ and NC groups (Fig. 7) paralleling the HOMA-beta scores, which further accounts for significant reductions in circulating serum insulin and insulin production. The healthier morphological appearance of pancreatic ß-cells in the FR10+STZ group than in the STZ group also confirms the stability of animals for a longer period of time. In fact, the STZ pancreatic islets revealed substantial damage, which was not noted in the FR10+STZ group. We speculate that fructose may compromise the effect of STZ by an unknown mechanism, resulting in the morphologically better appearance of the islets in the FR10+STZ group compared to the STZ group.

Recently, creatinine has been found to be a new risk factor for T2D and insulin resistance (Harita *et al.,* 2009). Creatinine is a metabolite derived from creatine, which is predominantly (>98%) found in skeletal muscle, the major site for insulin action and subsequent glucose disposal. Harita *et al.* (2009) proposed that if muscle mass is inversely proportional to insulin resistance but directly proportional to serum creatinine then serum creatinine could provide a conclusive measure to assess insulin resistance. Their study showed that lower serum creatinine level is a risk factor for the development of T2D in lean, non-obese Japanese males. More recently, Hjelmesæth *et al.* (2010) validated this finding as their research showed that the lower serum creatinine level can predict T2D in morbidly obese Caucasian patients. Our data showed a significantly lower ($p < 0.05$) serum creatinine level in diabetic rats as compared to NC rats coupled with significant increases in HOMA-IR in FR10+STZ rats emphasizing a state of insulin resistance in this group.

Hyperlipidemia is another factor directly linked to insulin resistance as high circulating lipid concentrations in the blood secrete humoral factors such as resistin and adiponectin that alter insulin sensitivity, leading to insulin resistance (Barnes and Miner, 2009, Trujillo and Scherer, 2006). Diets high in fructose cause hyperlipidemia and hypertension in various animal models (Hwang *et al.,* 1987) and have been linked over the years to insulin resistance (Tappy and Lê, 2010). This is partly because fructose metabolism cannot be controlled by insulin or leptin, which are important factors for the regulation of fat synthesis as well as energy intake (Basciano *et al*., 2005). Additionally, fructose has been shown to be more hyperlipidemic than both glucose and starch with increased levels of LDL, VLDL and total cholesterol in rats (Benado *et al.,* 2004). Several recent studies have been reported that higher

serum triglycerides alone or accompanied with low serum HDL-cholesterol is a risk factor for hepatic insulin resistance as well as T2D (Elliot *et al.,* 2002, Singla *et al.,* 2009). The significantly higher serum total cholesterol and triglyceride levels in the FR10+STZ group, but not in STZ group, compared to the NC group could be the reasons for higher insulin resistance in the FR10+STZ group compared to other groups.

In addition, higher level of liver-function enzyme e.g. alanine transaminase (ALT) and aspartate transaminase (AST) in serum are not only used for the identification of liver damage (Harris, 2005) but also for the hepatic insulin resistance, metabolic syndrome as well as T2D (Cho *et al.,* 2007). In a recent study, Cho *et al.* (2007) found a strong correlation between serum ALT level and HOMA-IR scores but not for the AST, and it has been indicated as a predictor of T2D in human subjects. The significantly ($p < 0.05$) higher level of serum ALT level in FR10+STZ group compared to NC group also suggest the higher level of insulin resistance in this group. Furthermore, as ALT is not commonly elevated in T1D, it can be a strong and independent predictor for T2D, (Harris, 2005) which is reflected in our model.

In conclusion, this study shows that the 10%-Fructose-fed-40 mg/kg BW-STZ-injected rat can be a new and alternative model for T2D due to its quick induction time that successfully induces both pathogeneses of T2D, insulin resistance and partial pancreatic ß-cell dysfunction. The model demonstrated a stable diabetic condition over an 11 weeks experimental period and can be used for both acute and chronic research studies as well as being useful for routine pharmacological screening of anti-type 2 diabetic materials. It is easy to develop, highly cost-effective and can be used by researchers world-wide, especially in developing countries, where funding and resources are limited. As neither special formulation of diets nor sophisticated instruments is required, this model can be developed in an animal laboratory with minimum facilities.

CHAPTER 3 Intervention Trial

3.1 Intervention trial background

One of the major aims of current research is to improve existing models or develop novel models that may better reflect the pathogenesis of type 2 diabetes (T2D). These models require to be validated through pharmacological screening of routine anti-diabetic drugs. The initial findings in chapter 3 revealed that the 10%-fructose-fed-STZ-injected rat was highly responsive to T2D drugs. To validate the initial findings, a more comprehensive intervention trial study was required by testing not only commonly employed anti-diabetic agents but also through the use of natural products believed to have anti-diabetic properties.

Despite the wide availability and range of existing anti-diabetic drug therapies, there is still an ever-growing need for better and/or alternative therapies to combat the rising numbers of global diabetic patients. Furthermore, the need for new therapies is increasing due to their cheaper cost, wider availability and to avoid the dissatisfactory symptoms and consequences of traditional drug therapies, including weight gain and hypoglycaemia or certain contraindications that may limit their use (Amin and Nagy, 2009; Tahrani *et al.,* 2010). Because T2D is characterised by 2 pathogeneses, insulin resistance and partial pancreatic β-cell dysfunction (Stumvoll *et al.,* 2005), these are two targets for disease control. Insulin resistance precedes the development of T2D by many years (Faccini *et al.,* 2001) with obesity being labelled the "single most important contributor" to insulin resistance (Kahn *et al.,* 2006; Tahrani *et al.,* 2010). The global prevalence of overweight and obesity are rapidly increasing not only in adults but also in young adolescents and children (Canbakan *et al.,* 2008). Obesity has not only been associated with insulin resistance but also with the development of T2D and other complications such as cardiovascular disease and coronary heart disease (Lavie *et al.,* 2008). At the same time, the reasons for the progression toward pancreatic β-cell dysfunction largely involve hyperglycemia and hyperinsulinemia. Achieving glycaemic control in those already suffering with T2D and preventing further pancreatic damage are thus other important therapeutic targets. For this reason, in the following experiment, an agent known as L-carnitine (CARN) with potential to reduce obesity, weight gain, cholesterol, and potential for anti-diabetic effects, as well as white mulberry leaf tea, with anti-oxidant pancreatic-protective and hypoglycaemic potential, were selected.

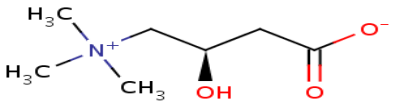

Figure 22: Chemical structure of L-Carnitine (www.bmrb.wisc.edu)

L-carnitine (ß-hydroxy-γ-N-trimethylammonium-butyrate) is ingested in the human diet from meat and dairy products, which accounts for 75% of adult carnitine requirements (Stanley, 2004). Besides the diet, carnitine can also be endogenously synthesized in the liver, kidneys and brain from two essential amino acids, methionine and lysine (Flanagan *et al*, 2010, Scaglia and Longo, 1999). L-carnitine is a compound that is vital for fat metabolism in the body as it facilitates ATP energy through the oxidation of fatty acids (Gómez-Amores *et al.,* 2007). L-Carnitine plays an essential role in β-oxidation, not only through the generation of ATP, but also through transportation of long-chain fatty acids from the cytosol into the mitochondrion (Amat di San *et al.,* 2008). Deficiencies in L-carnitine are therefore linked to the body's inability to utilise lipids as a source of fuel (Seccombe e*t al.,* 1987, Amat di San *et al.,* 2008).

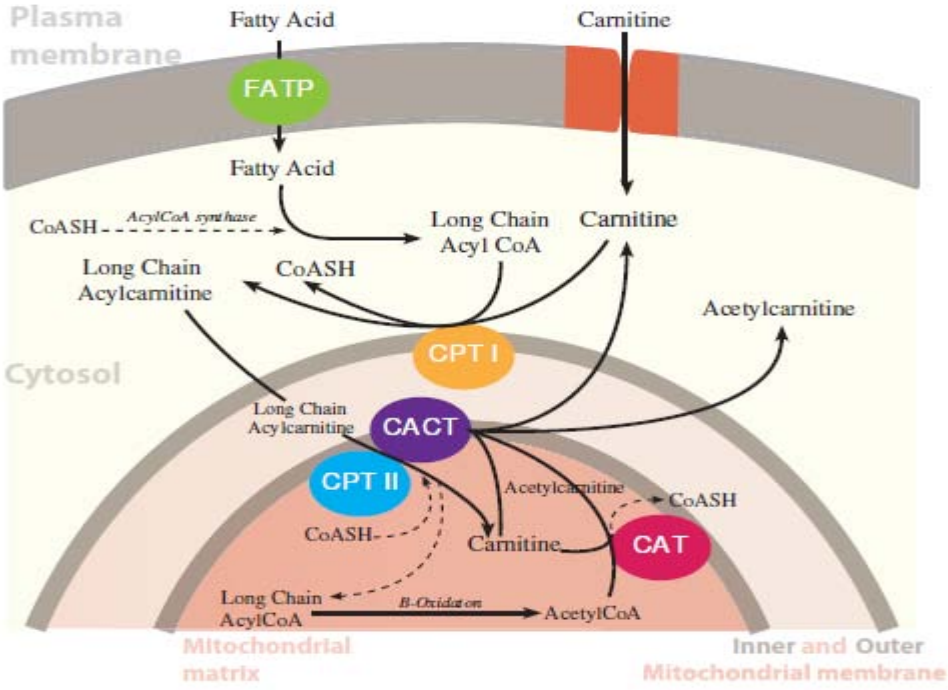

Figure 23: Transportation shuttling of long-chain fatty acids into the mitochondria for β-oxidation (Copied without permission from Flanagan *et al.*, 2010). FATP = Fatty acid

transport protein , CPT I = carnitine palmitoyltransferase 1, CPT II = carnitine palmitoyltransferase II, CACT = carnitine acylcarnitine translocase

Furthermore it is a potent antioxidant in the body as it is a scavenger of free radicals and protects cells from endogenous reactive oxygen species (ROS). ROS is a leading cause of oxidative stress, which results in partial pancreatic ß-cell dysfunction as well as hypertension and various other complications of the diabetic condition (Jingbo *et al.,* 2010). In addition, L-carnitine has been shown to reduce lipid peroxidation when orally administered (Clark *et al.,* 2007). Several studies have indicated its benefits in atherosclerotic rats and hypercholesterolaemic rabbits (Gómez-Amores *et al*., 2007). Furthermore, reports have indicated that L-carnitine reduces oxidation of LDL cholesterol. This finds benefit in diabetic patients due to the high level of oxidative stress found in these subjects (Motta *et al.,* 2006, Motta *et al.,* 2008). Oxidation of LDL cholesterol enhances the formation of "cholesterol-laden foam cells" (Malaguarnera *et al.,* 2009), which form a significant component in atherosclerotic plaques (Steinberg, 1997, Boullier *et al.,* 2001). These are both important disease complications of T2D and hence indicate its potential use as an anti-diabetic agent. L-carnitine deficient rats administered with L-carnitine resulted in the normalization of blood pressure, (Kraemer *et al.,* 2008) which is a necessary factor in diabetic patients suffering with elevated blood pressure and hypertension.

The liver is the primary site of L-carnitine metabolism, hence it may have a therapeutic benefit in diseased liver conditions. In fact, Yapar *et al.* (2007) found that L-carnitine administration significantly lowered the activity of functional liver enzymes AST and ALT in obese rats. Other studies revealed L-carnitine administration resulted in a reduction in insulin resistance in T2D patients when assessed using the euglycemic hyperinsulinemic clamp method (Capaldo *et al.,* 1991).

Low HDL and high serum triglyceride levels have been deemed a risk factor for the development of T2D (Singla *et al*., 2009). In 2004, studies conducted by Eskandari and co-workers found that diabetic rats induced by streptozotocin followed by L-carnitine treatment at a dose of 100 mg/kg body weight (BW) per day for 10 days showed both lower total cholesterol and triglyceride levels. They concluded that L-carnitine has hypotriglyceridemic and hypocholesterolemic potential and further studies must be done to evaluate its effectiveness as a potential agent for treatment of T2D. Importantly there is evidence that L-carnitine serves to enhance the glycolytic pathway – the major pathway of carbohydrate

metabolism. This is achieved as it enhances the activity of pyruvate dehydrogenase resulting in the reduction of acetyl CoA, which subsequently activates the glycolytic pathway (Mingrone *et al*,.1999). It can be suggested that through this mechanism L-carnitine can improve glucose disposal and hence lower hyperglycaemia/blood glucose. It is therefore of extreme importance and relevance to investigate L-carnitine as a therapeutic agent against T2D and determine all effects on parameters of T2D, including liver enzymes, kidney function, lipid profiling, body weight, food and fluid intake as well as serum proteins and uric acid levels.

On the other hand, white mulberry (*Morus alba*) has been used over the centuries in Traditional Chinese Medicine as a common agent to treat a variety of conditions including atherosclerosis, diabetes, cancer as well as for boosting the immune system and offering protection to nerves through potent antioxidant activity (Butt *et al.,* 2008).

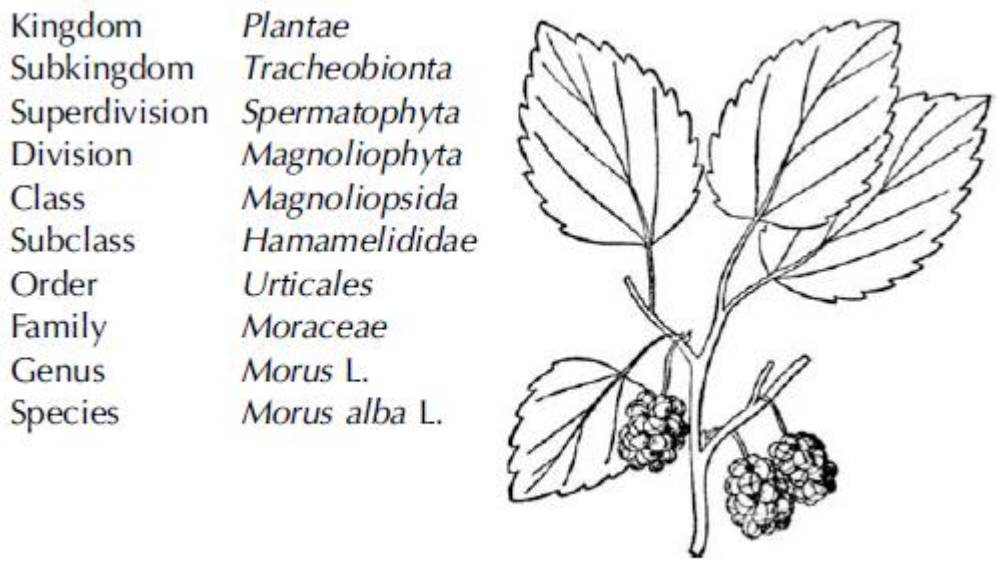

Figure 24: Classification and identification of White Mulberry (*Morus alba)* (Copied without permission from Butt *et al.*, 2008)

With regards to diabetes, the root and bark are often used to reduce hyperglycemia (high blood sugar) (Bantle and Slama, 2006). Different parts of the Mulberry plant (fruit, bark, leaf and root) have drawn interest in their role to treat diabetes as a result of the different chemical components found in the various plant constituents. One such component, 1-deoxynojirimycin (DNJ), found in both the leaves and the bark, is a polyhydroxylated piperidine alkaloid known to be a potent α-glycosidase inhibitor (Oku *et al.*, 2006) which aids

to slow down the uptake of carbohydrates and hence help manage blood glucose levels. Kimura and coworkers (2007) found that a single dose of DNJ in humans of 0.8 and 1.2g significantly lowered non-fasting blood glucose levels and insulin secretion. Whilst the anti-diabetic effects of the root and bark has been known for centuries, only recently, the leaves used in the brewing of tea has gained a great deal of interest as a potential anti-diabetic drink (Butt *et al*., 2008). Studies conducted by Hansawasdi and Kawabata (2006) showed that 1.0% mulberry tea solution had inhibitory effects against sucrose, maltase and α-glucosidases, preventing the digestion of carbohydrates and delaying their absorption, ultimately reducing blood glucose levels. White mulberry contains a significant concentration of anti-oxidative compounds. Mulberry leaves are found to contain phenolic compounds, flavonoids. Mulberry leaves prevent lipid peroxidation in diabetic patients by reducing catalase activity and increasing glutathione concentrations (Andallu and Varadacjaryulu, 2003). Mulberry leaf extracts inhibit oxidation of LDL cholesterol and thus find benefit in treating atherosclerosis (Enkhmaa *et al*., 2005). In LDL deficient mice, consumption of mulberry leaves lowered LDL oxidation, resulting in a reduction of atherosclerotic lesion areas (Katsube *et al*., 2006). The tea has not yet been tested in animal models and is highly relevant to determine its anti-diabetic effects. Furthermore, due to its potent antioxidant activity, it may serve to offer protection against later diabetic complications (such as neuropathy and cardiomyopathy) and requires investigation on other diabetic parameters.

3.2 Materials and method

3.2.1 Reagents and materials

STZ (>98%) (Sigma-Aldrich) was purchased from Capital Lab Supplies cc. Durban, South Africa. Glucostrips, and Fructose (Nature's Choice™ Wholefood specialists, Meyerton, South Africa 1960) were purchased from a local pharmacy. L-carnitine was purchased from Sunrise Chemical co. (South Africa). White mulberry tea was purchased from Beautique Thai (Thailand). A glucometer (GlucoPlus Inc, Quebec, Canada, measuring up to 600 mg/dl) was used for measuring fasting and non-fasting blood sugar levels. Serum analysis was conducted using an Automated Chemistry Analyzer (Labmax Plenno, Labtest, Brazil) according to manufacturer's instructions.

3.2.2 Animals

Thirty five (6 weeks of age) male Sprague-Dawley rats (mean similar weight 191.88g ± 16.40g) were procured from the Biomedical Resource Unit (BRU) at Westville Campus from the University of KwaZulu-Natal, South Africa. Animals were randomly subdivided into 5 groups of 7 rats in each group as follows: Normal Control (NC), Diabetic/Streptozotocin control (FR10+STZ), Mulberry Tea Low (0.25%) (FR10+STZ+MTL), Mulberry Tea High (0.5%) (FR10+STZ+MTH) and L-Carnitine (FR10+STZ+CARN). Two rats per poly-carbonated cage were housed in a temperature and humidity controlled room with a set 12 hour light-dark cycle. The rats were fed a commercially available rat chow diet *ad libitum* throughout the entire 9 week experimental period. The NC group was supplied with normal drinking water *ad libitum* whilst all other groups were supplied with a 10% fructose solution in drinking water for 2 weeks only to induce insulin resistance. Hereafter all groups except the MT groups were supplied with normal drinking water for the remainder of the experiment. The animals were maintained according to the rules and regulations of the University of KwaZulu-Natal (UKZN) Animal Ethics Committee (Ethical approval number: 029/11/Animal).

3.2.3 Induction of diabetes

STZ was dissolved in a citrate buffer (pH 4.4). The solution was then filtered through a sterilized 0.45 millipore filter. Each of the fructose-fed groups were injected intraperitoneally in the fasted state with a low dose STZ injection (40 mg/kg BW) whilst the normal control group (NC) received a vehicle citrate buffer (pH 4.4) injection only.

Non-fasting blood glucose (NFBG) levels of all animals was measured 1 week after STZ injection. Blood was taken from the rat tail veins using a 25G needle and FBG was measured using a portable glucometer (GlucoPlus Inc, Quebec, Canada). Animals with a non-fasting blood glucose (NFBG) level > 300 mg/dl were considered diabetic. NFBG levels were measured (Week 1-9) weekly. The rats were firstly fasted for a 12 hour period, after which food was returned *ad libitum* for 3 hours and then subsequent NFBG was measured. During the fasted period, all groups received normal drinking water and the amount consumed after the 12 hour period was recorded. During the 3 hour feeding period prior to NFBG measurements, intervention materials were administered returned, whilst the control groups continued to consume normal drinking water throughout this period.

3.2.3.1 Food and fluid intake, body weight and intervention materials

Daily food and fluid intake were monitored and weekly body weight changes were measured during the entire experimental period. Mulberry tea was prepared in the following concentrations: 0.25% and 0.5%. The tea was brewed for 10 minutes and cooled to room temperature. Mulberry tea was supplied *ad libitum* during week 4-8 of the intervention trial. L-Carnitine was prepared by dissolving in distilled water at a concentration of 250 mg/ml. L-carnitine was administered to the CARN group at a concentration of 500 mg/kg BW once daily in the morning during week 4-8 of the intervention trial.

3.2.4 Oral glucose tolerance test

After an overnight fast (12 hours), rats were orally dosed with a D-glucose solution (2.0 g/kg BW) at week 8 of the experimental period. Glucose concentrations were subsequently measured by blood collection from the tail veins at 0 (just prior to oral glucose dosing), 30, 60, 90 and 120 minutes after oral dosing and blood glucose levels monitored with a glucometer.

3.2.5 Collection of blood, liver, pancreas, kidneys, heart and brain

At the end of the experimental period animals were fasted for 14 hours prior to euthanasia by halothane anaesthesia. Animals were weighed before euthanasia after which blood, liver, heart, kidneys and pancreas were collected. Blood was collected through cardiac puncture using a 21G needle and fasting blood glucose levels were immediately measured using a portable glucometer. Blood samples were subsequently placed in heparin tubes and allowed to coagulate on ice for approximately 3 hours and then centrifuged at 3000 rpm for 15 minutes. Hereafter, serum was separated into labeled microtubes and stored at -30˚C for further analysis. Liver, kidney, brain and heart samples were washed in cold 0.9% saline solution, wiped dry with filter paper, weighed on an analytical balance and preserved at -30˚C for further analysis. The hypothalamus was frozen in liquid nitrogen and stored for further analysis. Part of the pancreas sample was immediately fixed in 10% neutral buffered formalin solution for histopathological examinations, which was replaced weekly during the entire preservation period. The remainder of the pancreas was preserved at -30˚C for further analysis.

3.2.6 Analytical methods

Liver glycogen concentration was measured photometrically by using the Phenol-Sulphuric acid method as described by Lo *et al*. (1970). Serum insulin was analyzed using an ultra sensitive rat insulin ELISA kit (Mercodia AB, Uppsala, Sweden) using a multi plate ELISA reader (Biorad-680, BIORAD Ltd., Japan).

Serum lipid profiles, serum creatinine and liver function enzymes (AST and ALT) were measured using an automated chemistry analyzer (Labmax Plenno, Labtest, Brazil). Total cholesterol was measured directly in the serum according to the following principle:

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{Cholest-4-en-one} + H_2O_2.$$

$$H_2O_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{Quinonemine} + 4H_2O.$$

Quinonemine is maximally absorbed at 500 nm. The intense red colour generated is directly proportional to the cholesterol concentration in the sample. LDL cholesterol was quantitatively precipitated after centrifugation. Triglycerides are measured in a similar manner to total cholesterol to yield quinonemine. The red colour that is produced is directly proportional to triglyceride concentration.

Creatinine was measured according to the following test principle:

$$\text{Creatinine} + \text{alkaline picrate} \longrightarrow \text{Creatinine picrate}$$

This yields an intense red colour. This red colour is directly proportional to the concentration of creatinine at 510 nm.

Liver enzyme ALT was measured according to the following test principle:

$$\text{L-Alanine} + \text{ketoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L-glutamate}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}$$

NADH oxidation at 340 nm is directly proportional to the ALT activity in a sample. AST is measured in an almost identical manner:

$$\text{L-Aspartate} + \text{ketoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L-glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}$$

Fructosamine is formed when glucose binds to amino acids in the blood and rearranges molecularly to form fructosamine. In an alkaline pH, fructosamine reduces blue tetrazolium to purple formazan which can be directly measured at 530 nm.

Uric acid can be measured in serum in the following 2 step reaction:

$$\text{Uric acid} + 2H_2O + O_2 \xrightarrow{\text{uricase}} \text{Allantoin} + CO_2 + H_2O_2.$$

$$2H_2O_2 + \text{DHBS} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{Quinonemine} + 4H_2O.$$

Quinonemine yields an intense red quinonemine measured between 490-540 nm. This is directly proportional to the concentration of serum uric acid in a sample.

Total proteins and serum albumin are measured by the principle that in alkaline conditions, copper ions react with peptide bonds in proteins to yield a purple colour that is maximally absorbed at 545 nm and is directly proportional to the concentration of proteins and albumin in a serum sample.

3.2.7 Liver glycogen content

Liver glycogen content was measured photometrically according to the Phenol-Sulphuric acid Method developed by Lo *et al.* (1970). Liver samples of ≤ 1.0g were measured and placed at the bottom of a screw-capped pyrex tube using forceps and then placed on ice. The sample was then immersed in 1.5ml of 30% KOH saturated with Na_2SO_4. This was then placed in a boiling water bath for 20-30 minutes. The sample was then removed and allowed to cool on ice. 2ml of 95% ethanol was then added and placed on ice for a minimum of 30 minutes. Samples were centrifuged at 840 x g (2812 rpm) for 25-30 minutes. The supernatant was aspirated and the glycogen precipitate was dissolved in 3.0ml distilled water. 50µl of this solution was transferred to a clean test tube containing 450µl distilled water. This was performed in duplicate. Glycogen standards were prepared from a stock solution of 1 mg/ml glycogen (Oyster, Type II, SIMGA-ALDRICH, USA) at concentrations of 10, 20, 40, 80, 160, 320 and 640µg/ml. To both the standards and samples, 0.5ml of 5% phenol was added to each test tube, followed by the rapid addition of 2.5ml 96-98% H_2SO_4. This was left to stand at room temperature for 10 minutes, after which the absorbance was read in a photometer at 490nm. A solution of distilled water served as the blank. The concentration of liver glycogen

was thereafter calculated from a liver glycogen standard curve. This experiment was performed in triplicate.

3.2.8 Serum insulin analysis

Rat serum analysis was performed using a Ultrasensitive Rat Insulin ELISA kit from Mercodia (Uppsala, Sweden). This kit was supplied with a method for the quantitative determination of serum insulin. It makes use of a direct technique whereby 2 monoclonal antibodies act against different antigenic sites on the insulin molecule. Insulin contained in the rat serum reacts with a peroxidase-conjugated anti-insulin antibody during incubation. The several washing steps removed any unbound enzyme-labelled antibodies. The bound conjugate is then detected through the reaction with 3,3',5,5'-tetramethylbenzidine. This reaction is then stopped by the addition of acid giving rise to a yellow colour that is read photometrically at 450nm. This experiment was performed through the development of a serum insulin standard curve using 50µl of a range of calibrators from Calibrator 0-7. In separate wells, 50µl of serum sample was added after which 50µl of enzyme conjugate was added to all wells. This was incubated on a shaker at 700-900rpm for 2 hours at room temperature. Hereafter, the solution was aspirated followed by 6 wash steps of 350µl of 21x wash buffer. At the last wash step, the plate was inverted and tapped firmly against absorbent paper towel. 200µl of Substrate TMB was added to all wells. This was incubated for 30 minutes to develop a blue colour. After 30 minutes, a stop solution comprised of acid was added to all wells in 50µl aliquots. This was shaken for 5 seconds to ensure adequate mixing of Substrate TMB and the stop solution. The absorbance was read in triplicate using a multiplate reader ((Biorad-680, BIORAD Ltd., Japan) at 450nm.

3.2.9 Statistical analysis

All data are presented as mean ± SD. The data were analyzed by a statistical software package (SPSS version 18) using the Tukey-Kramer multiple range *post-hoc* test. The values were considered significantly different at $p < 0.05$.

3.3 Results

3.3.1 Food intake during intervention trial

Figure 25 represents the mean food intake (g) per animal per day during the intervention trial period. The NC group displayed significantly lower food intake compared to all groups, whilst the FR10+STZ+MTL group displayed significantly higher food intake compared to all other groups. There were no statistical differences of the food intake between the FR10+STZ (50.72 ± 4.62g), FR10+STZ+MTH (50.81 ± 5.76g) and FR10+STZ+CARN (49.38 ± 8.08g) groups.

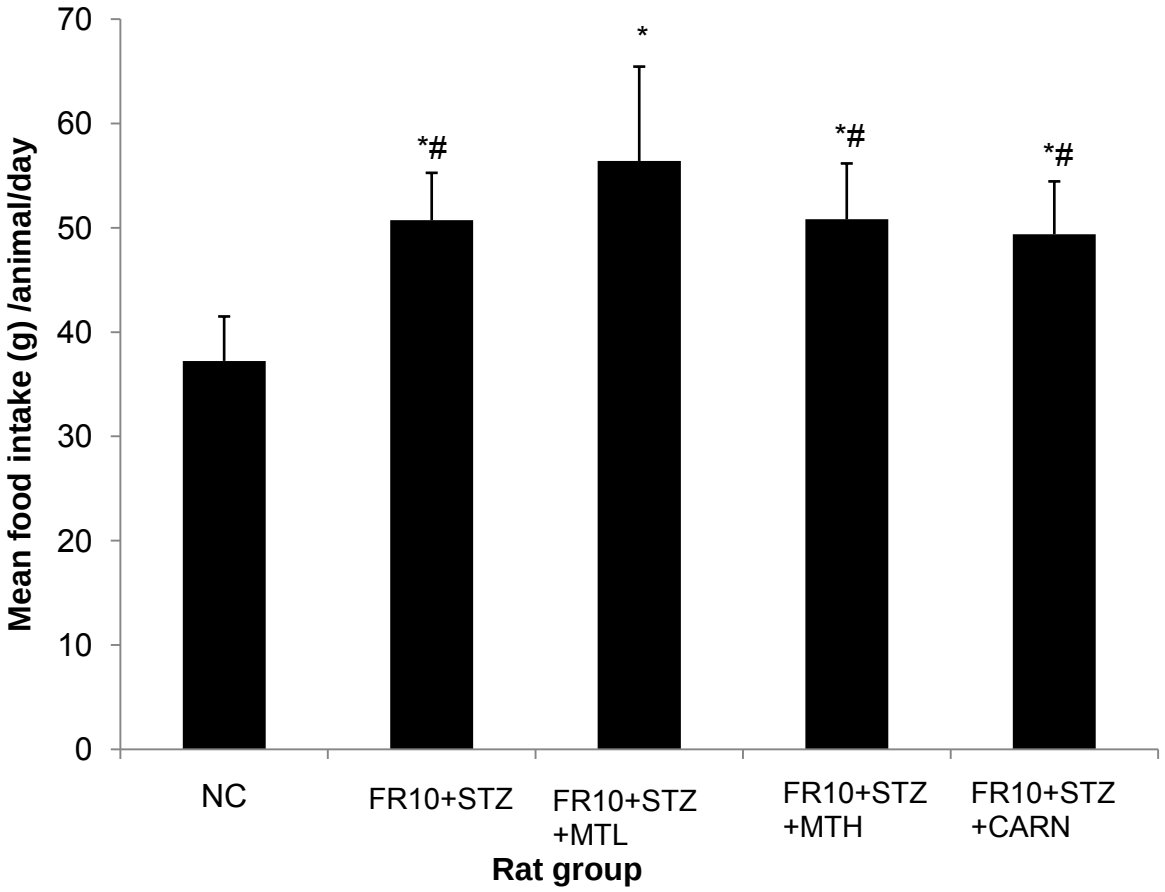

Figure 25: Mean food intake (g) per animal per day during the intervention period. *p < 0.05 vs. NC, #p < 0.05 vs. FR10+STZ+MTL. (Tukey-Kramer multiple range *post-hoc* test).

3.3.2 Mean fluid intake during intervention trial

Figure 26 shows the mean fluid intake (ml) per animal per day during the invention trial period. The graph shows the NC group displayed a significantly lower fluid intake compared to all other groups, whilst the FR10+STZ+MTL (198.24 ± 17.46ml) group displayed significantly higher fluid intake compared to all groups. No statistical differences were noted between FR10+STZ (179.84 ± 27.99ml) and FR10+STZ+MTH (175.37 ± 21.11ml), however, FR10+STZ+CARN (163.83 ± 26.08ml) displayed significantly lower fluid intake compared to the FR10+STZ group.

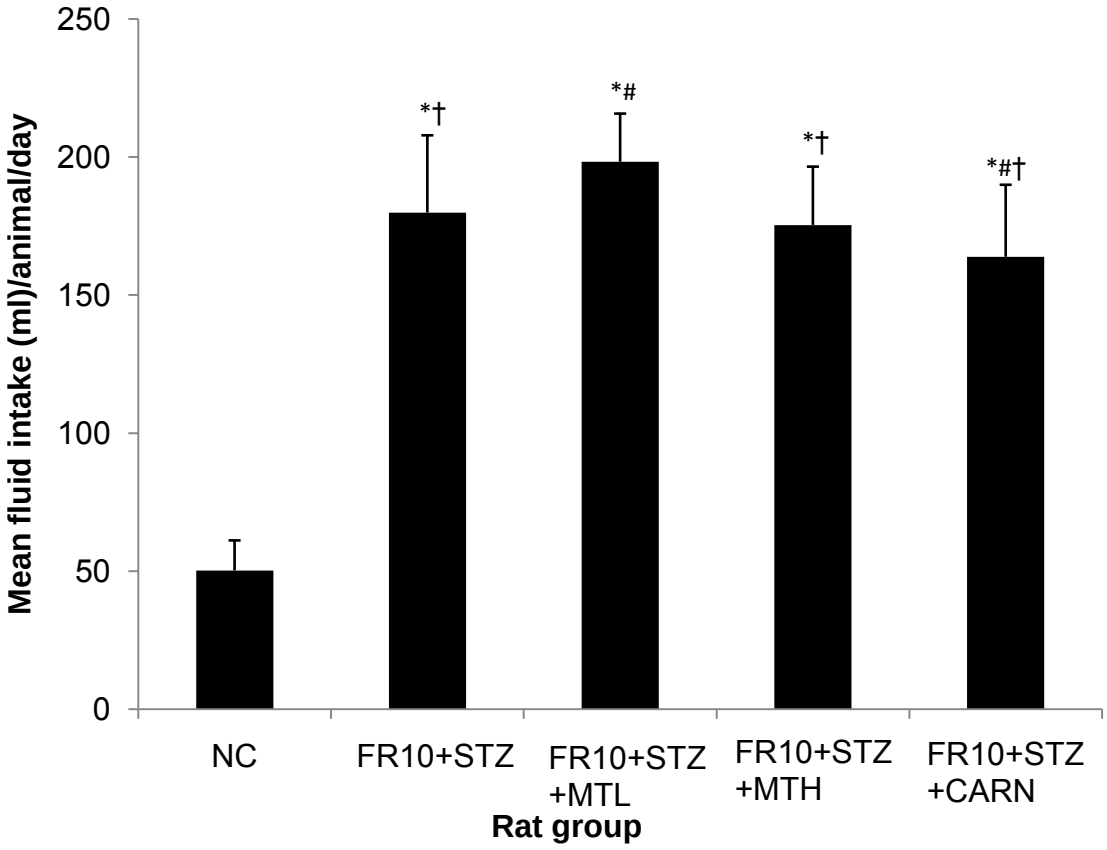

Figure 26: Mean fluid (ml) intake per animal per day during the intervention trial period. *p < 0.05 vs. NC, #p < 0.05 vs. FR10+STZ, †p < 0.05 vs. FR10+STZ+MTL. (Tukey-Kramer multiple range *post-hoc* test).

3.3.3 Mean body weight over the experimental period

Figure 27 represents the mean body weight over the 9 week experimental period. The first 3 weeks reveal no significant changes in body weight until the induction of diabetes through the intraperitoneal injection of streptozotocin. One week after the streptozotocin injection and all experimental groups displayed significant reduction in body weight compared to the NC group. This weight loss is a common symptom of streptozotocin and is quickly recovered. Throughout the intervention period, all experimental groups including FR10+STZ displayed significantly reduced body weight compared to the NC group, however there were no statistical differences among the groups. FR10+STZ+MTH and FR10+STZ+CARN displayed non-significantly reduced body weight compared to FR10+STZ, with FR10+STZ+MTL having a non-significantly increased body weight compared to FR10+STZ. FR10+STZ+CARN displayed the lowest body weight over the experimental period.

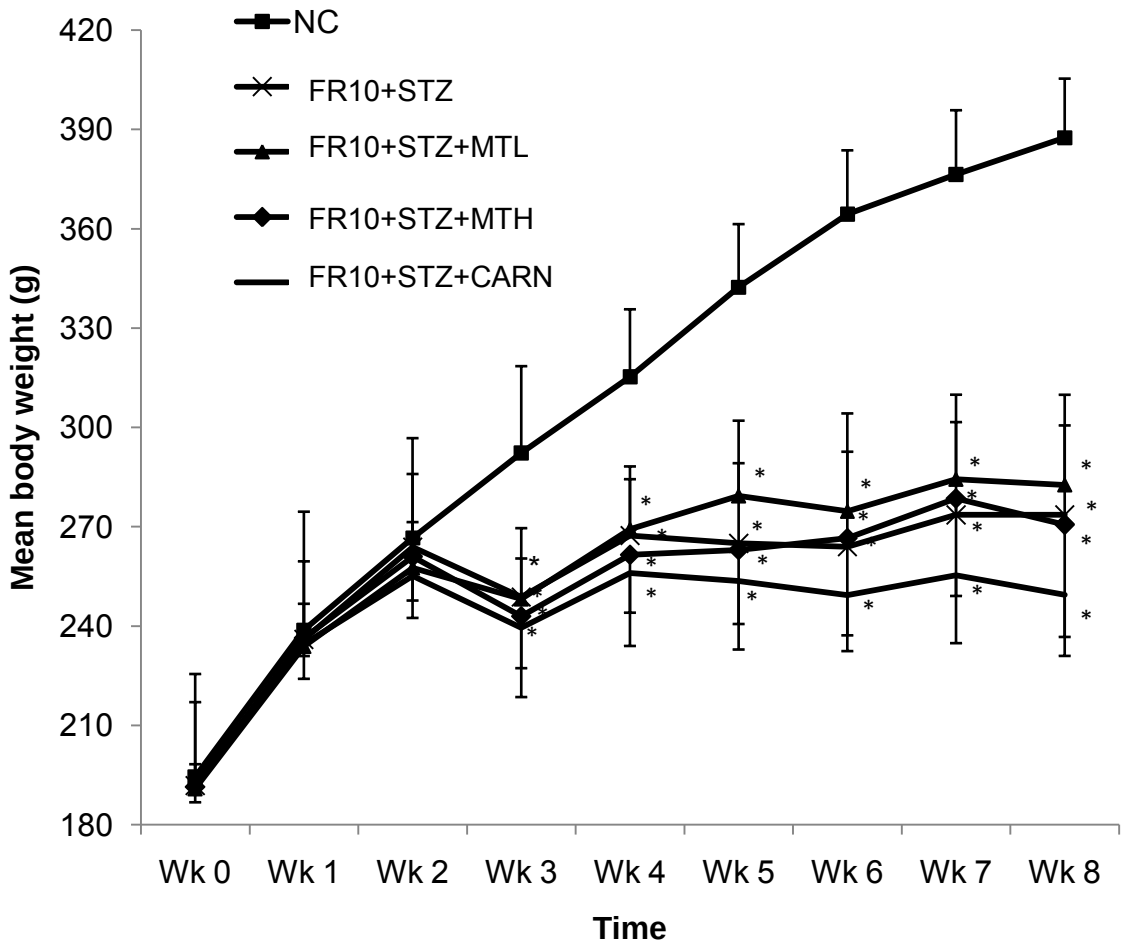

Figure 27: Mean body weight (g) over the 9 week experimental period.*$p < 0.05$ vs. NC. (Tukey-Kramer multiple range *post-hoc* test).

3.3.3.2 Mean body weight gain

Figure 28 depicts the mean body weight gain over the entire 9 week experimental period. The NC group displayed significantly higher body weight gain compared to all groups. The experimental groups showed no significant differences compared to the FR10+STZ (24.8 ± 18.79g) group, however both MT groups were non-significantly higher, whilst FR10+STZ+CARN (10.00 ± 15.49g) was non-significantly lower than FR10+STZ. FR10+STZ+CARN displayed the lowest overall weight gain and FR10+STZ+MTL (34.5 ± 21.60g) displayed the highest overall weight gain of the diabetic groups.

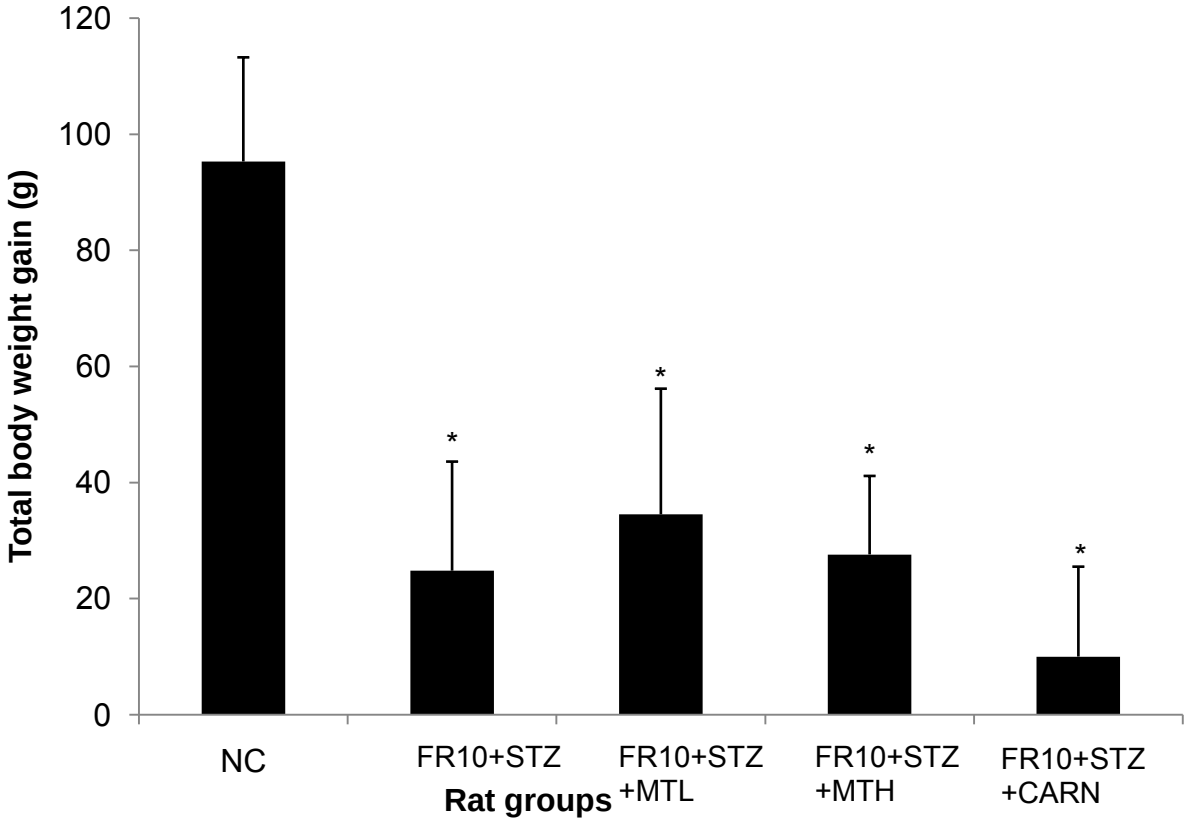

Figure 28: Mean weight gain over the experimental period. *p < 0.05 vs. NC. (Tukey-Kramer multiple range *post-hoc* test).

3.3.4 Non-fasting blood glucose

3.3.4.1 Non-fasting blood glucose (mg/dl) of FR10+STZ+MTL during intervention trial.

Figure 29 displays the NFBG of NC, FR10+STZ and FR10+STZ+MTL during the intervention trial period. FR10+STZ and FR10+STZ+MTL displayed significantly higher NFBG compared to NC. The NC group displayed extremely stable NFBG over the 4 week period with a mean NFBG ranging between 106 mg/dl to 109 mg/dl. The FR10+STZ group was not significantly different from FR10+STZ+MTL group over the experimental period with NFBG ranging between 562.60 - 599.40 mg/dl and 563.67-600.00 mg/dl respectively.

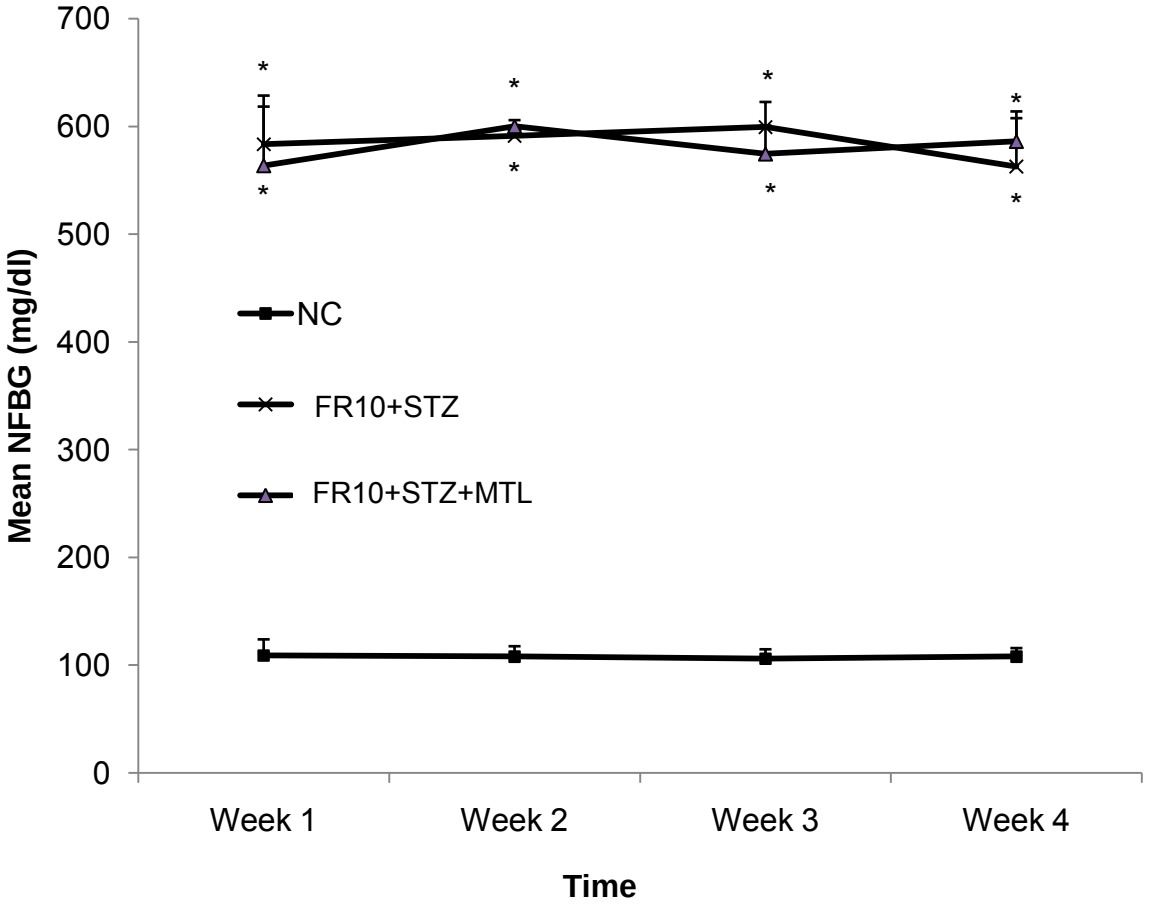

Figure 29: Non-fasting blood glucose (mg/dl) of FR10+STZ+MTL group over intervention trial. *$p < 0.05$ vs. NC. (Tukey-Kramer multiple range *post-hoc* test).

3.3.4.2 Non-fasting blood glucose (mg/dl) of FR10+STZ+MTH during intervention trial

Figure 30 displays the NFBG (mg/dl) of the FR10+STZ+MTH group during the 4 week intervention trial. FR10+STZ and FR10+STZ+MTH displayed significantly higher NFBG compared to NC. The NC group displayed a mean NFBG ranging between 106 mg/dl to 109 mg/dl. The FR10+STZ group was not significantly different from FR10+STZ+MTH group over the experimental period with NFBG ranging between 562.60 - 599.40 mg/dl and 572.80 – 592.00 mg/dl respectively.

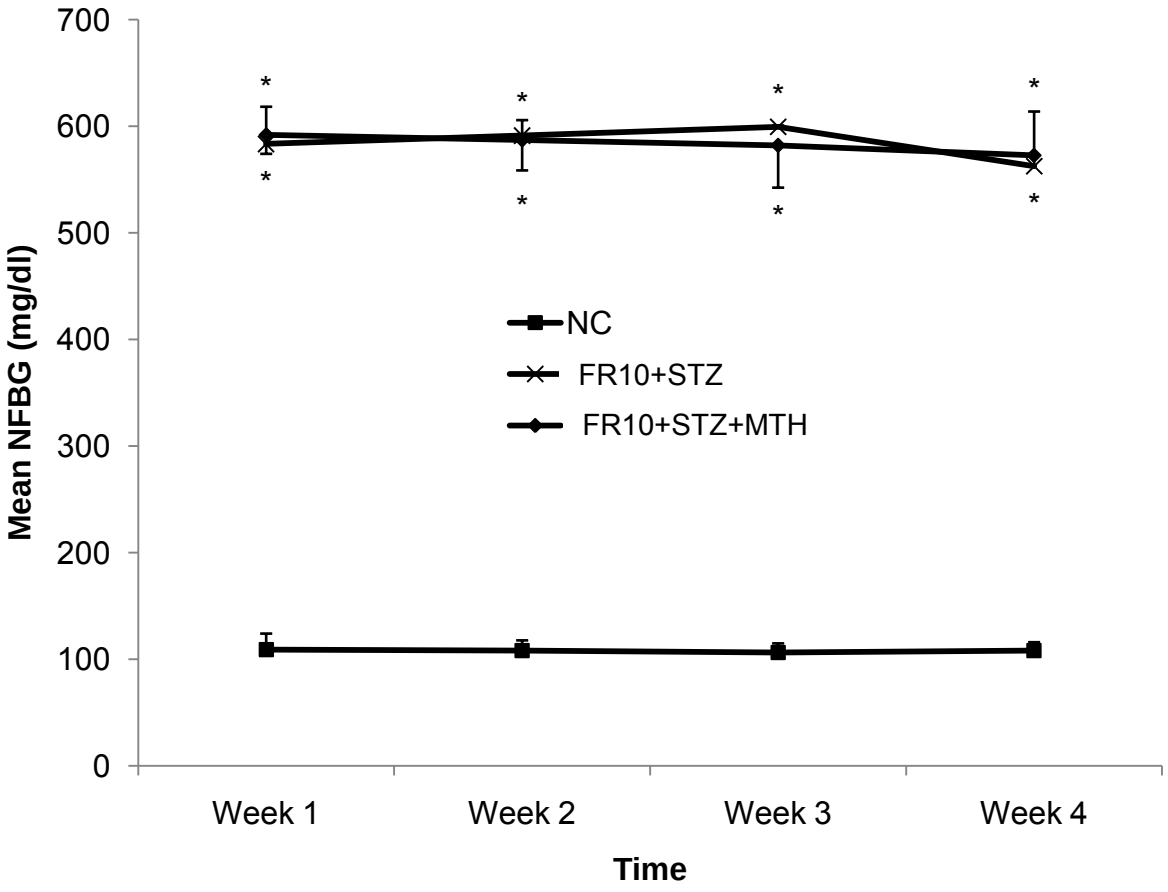

Figure 30: Non-fasting blood glucose (mg/dl) of FR10+STZ+MTH during the intervention trial period. *$p < 0.05$ vs. NC. (Tukey-Kramer multiple range *post-hoc* test).

3.3.4.3 Non-fasting blood glucose (mg/dl) of FR10+STZ+CARN during intervention trial.

Figure 31 displays the NFBG (mg/dl) of the FR10+STZ+CARN group during the 4 week intervention trial. FR10+STZ and FR10+STZ+CARN displayed significantly higher NFBG compared to NC. The NC group displayed a mean NFBG ranging between 106 mg/dl to 109 mg/dl. The FR10+STZ group was not significantly different from the FR10+STZ+CARN group over the experimental period with NFBG ranging between 562.60 - 599.40 mg/dl and 552.30 - 477.50 mg/dl respectively. Important to note is that no other experimental group except FR10+STZ+CARN displayed a trend pattern in the weekly NFBG measurements. FR10+STZ+CARN was the only group to display a consistent reduction each week in NFBG. Larger standard deviations can be noted on the FR10+STZ+CARN group compared to all other groups as one animal in this group displayed significant reductions from 421 mg/dl in week 1 to 179 mg/dl in week 4.

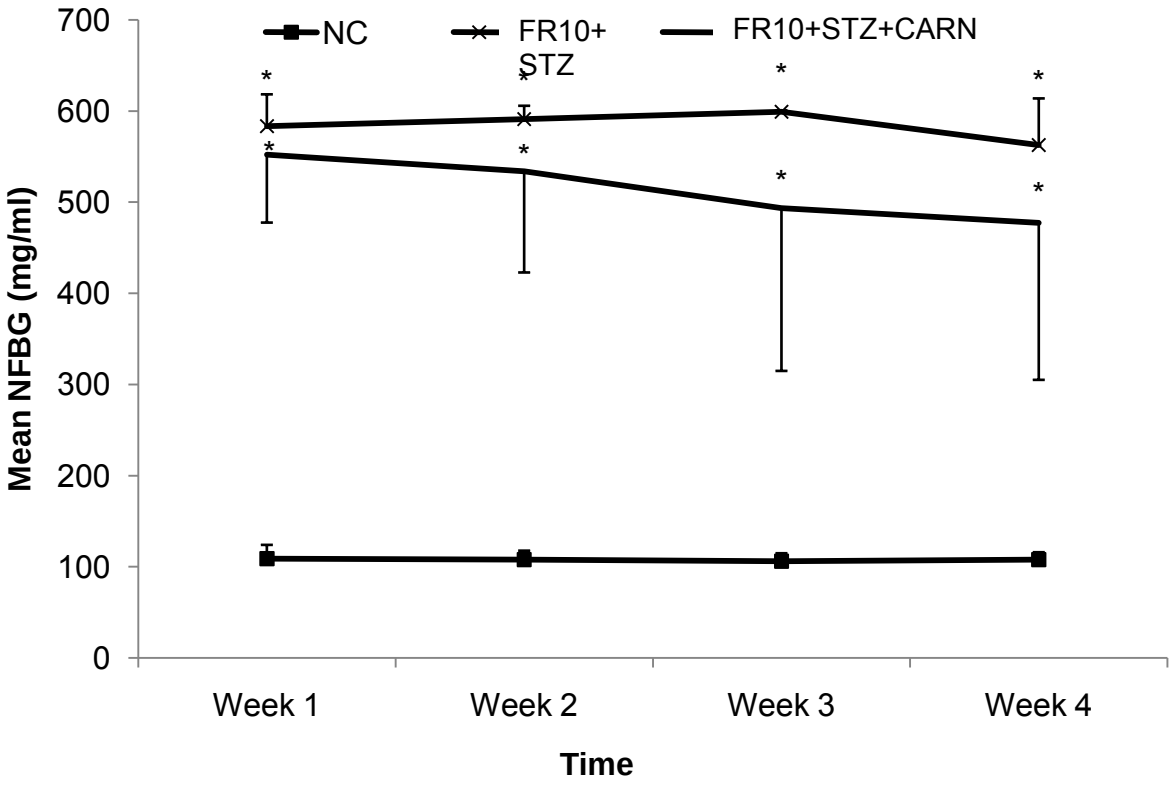

Figure 31: Non-fasting blood glucose (mg/dl) of FR10+STZ+CARN during the intervention trial period. *p < 0.05 vs. NC. (Tukey-Kramer multiple range *post-hoc* test).

3.3.5 Oral glucose tolerance test

Figure 32 represents the OGTT during week 8 of the experimental period. Just prior to dosing 2g/kg BW D-glucose (time 0 minutes), all groups except FR10+STZ+CARN had significantly higher FBG compared to NC. At time 0 minutes, FR10+STZ+CARN displayed significantly lower FBG than the FR10+STZ+MTL group. All groups peaked at 30 minutes after oral dosing. All groups were significantly higher than the NC group between 30-120 minutes, and FR10+STZ+CARN also displayed significantly lower BG after 120 minutes compared to the FR10+STZ+MTL group. None of the experimental groups demonstrated a BG less than 200 mg/dl after 2 hours, indicating that the diabetic condition had not been reversed. The NC group ranged from 87.90 - 129.70 mg/dl, FR10+STZ from 301.20 -397.00 mg/dl, FR10+STZ+MTL from 439.70 - 457.80 mg/dl, FR10+STZ+MTH from 305.80 - 468.20 mg/dl and FR10+STZ+CARN from 231.80 – 387.00 mg/dl.

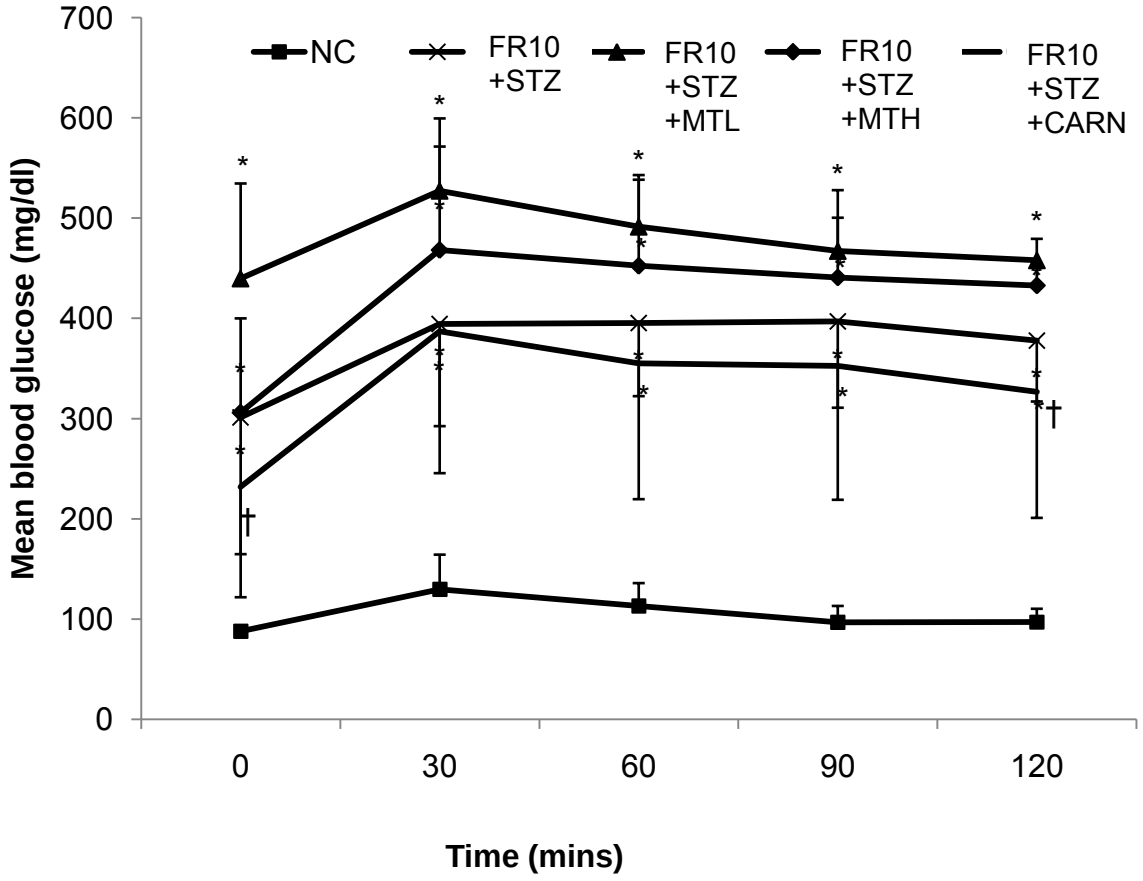

Figure 32: Oral glucose tolerance test over a 2 hour period. *$p < 0.05$ vs. NC., †$p < 0.05$ vs. FR10+STZ+MTL. (Tukey-Kramer multiple range *post-hoc* test).

3.3.6 Liver weight, relative liver % and liver glycogen

Table 6 shows the liver weight, relative liver weight (%) and liver glycogen (mg/g liver tissue). No significant differences were noted among groups for liver weight, except for the FR10+STZ+CARN group, which displayed a significantly lower liver weight compared to the NC group. All groups, however, displayed a significantly higher relative liver weight and liver glycogen compared to the NC group, yet there were no statistical differences amongst the experimental groups themselves.

Table 6: Liver weights and liver glycogen levels in different animal groups at the end of the experimental period.

Rat group	Normal Control (NC)	Diabetic Control (FR10+STZ)	Mulberry Tea Low (FR10+STZ +MTL)	Mulberry Tea High (FR10+STZ +MTH)	Carnitine (FR10+STZ +CARN)
Liver weight (g)	11.81 ± 0.8	10.66 ± 1.3	10.51 ± 1.1	10.33 ± 1.8	8.99 ± 0.9*
Relative liver weight (%)	3.05 ± 0.1	3.90 ± 0.3*	3.72 ± 0.1*	3.80 ± 0.3*	3.61 ± 0.4*
Liver glycogen (mg/g tissue)	4.66 ± 3.61	11.97 ± 0.16*	11.46±1.25*	10.88 ± 2.67*	11.50 ± 0.63*

Data are shown as mean ± SD of 7 animals. *$p < 0.05$ vs. NC. (Tukey-Kramer multiple range *post-hoc* test).

3.3.7 Serum insulin

Figure 33 shows the serum insulin (pmol/L) concentration in different rat groups at the end of the experimental period. All diabetic groups displayed significantly lower serum insulin compared to the NC group, yet the experimental groups demonstrated a minor, albeit, non-significant increase in serum insulin compared to the FR10+STZ group. FR10+STZ+CARN demonstrated the highest, although non-significant, concentration of serum insulin in the experimental groups, with FR10+STZ+MTH showing a higher serum insulin compared to FR10+STZ+MTL.

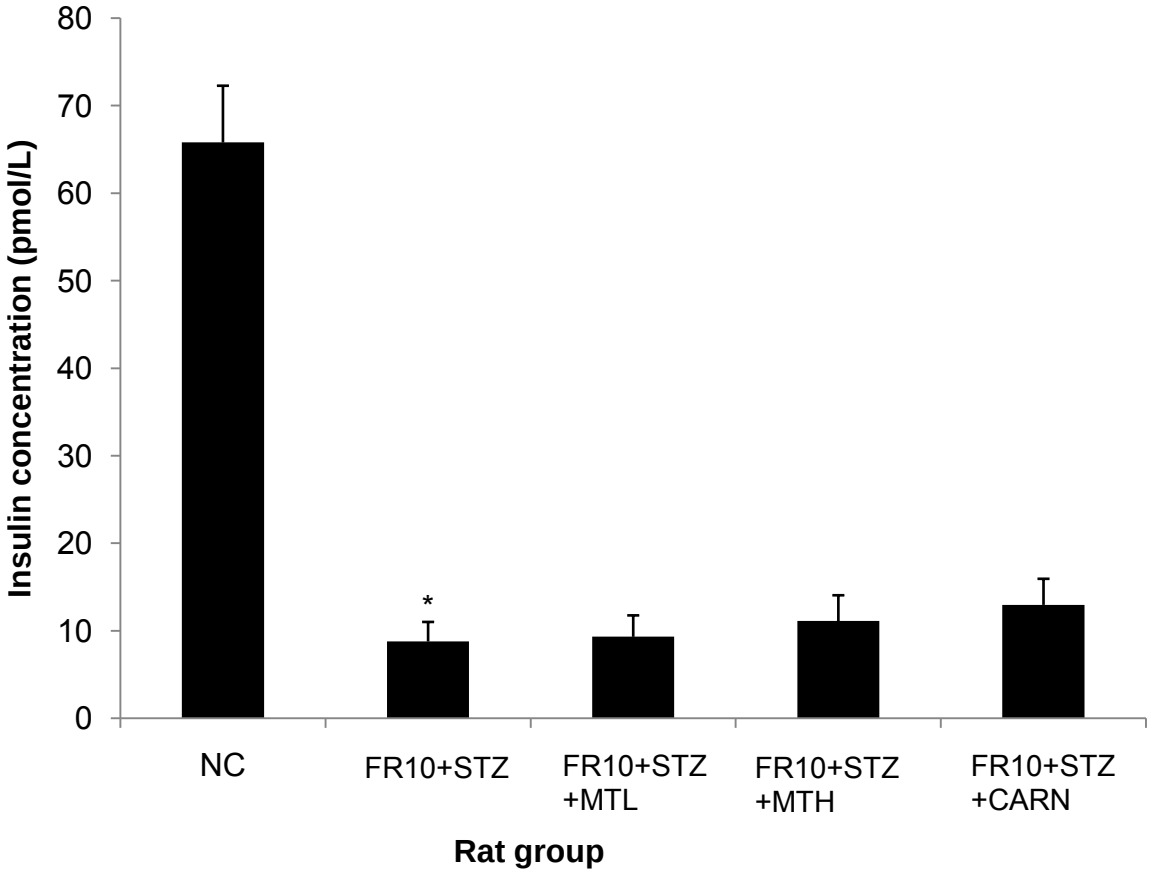

Figure 33: Serum insulin (pmol/L) of the experimental groups after the experimental period. *$p < 0.05$ vs. NC. (Tukey-Kramer multiple range *post-hoc* test).

3.3.8 Serum lipid profile

Table 7 represents the serum lipid profile after the experimental period. FR10+STZ+MTH and FR10+STZ+CARN displayed significantly lower total cholesterol compared to FR10+STZ and FR10+STZ+MTL, yet none were statistically different from the NC group. FR10+STZ+MTL displayed significantly higher HDL cholesterol compared to FR10+STZ, but no other statistical differences were noted for this test. No statistical differences in LDL cholesterol were noted amongst the groups, however, FR10+STZ and FR10+STZ+MTL displayed significantly higher triglycerides compared to the NC group, whereas FR10+STZ+MTH and FR10+STZ+CARN were not statistically different from NC. FR10+STZ+CARN was significantly lower than the FR10+STZ group.

Table 7: Serum lipid profile of the animal groups at the end of the experimental period.

Rat groups/ serum lipids	Normal Control (NC)	Diabetic Control (FR10+STZ)	Mulberry Tea Low (FR10+STZ+ MTL)	Mulberry Tea High (FR10+STZ+ MTH)	Carnitine (FR10+STZ+ CARN)
			mg/dl		
Total cholesterol	75.14 ± 8.71	79.14 ± 4.85	81.24 ± 13.01	63.60 ± 5.82$^{\#\dagger}$	63.33 ± 8.71$^{\#\dagger}$
HDL cholesterol	21.71 ± 4.54	17.80 ± 1.77	24.50 ± 2.50$^{\#}$	18.40 ± 5.90	18.33 ± 3.14
LDL cholesterol	41.20± 9.09	41.50 ± 6.31	36.81 ± 8.90	30.56 ± 4.63	33.40 ± 5.02
Triglycerides	61.14 ± 11.2	99.20 ± 20.2*	99.67 ± 25.3*	73.20 ± 9.37	58.00 ± 16.4$^{\#}$

Data are shown as mean ± SD of 7 animals.*p < 0.05 vs. NC, #p < 0.05 vs. FR10+STZ, †p < 0.05 vs. FR10+STZ+MTL. (Tukey-Kramer multiple range *post-hoc* test).

3.3.9 Liver function enzymes and serum creatinine

Table 8 represents the liver function enzymes of the animal groups at the end of the experimental period. No statistical differences were noted for AST amongst the groups. FR10+STZ and FR10+STZ+MTH displayed significantly higher ALT concentrations compared to the NC group. No statistical differences in serum creatinine were noted amongst the experimental groups.

Table 8: Serum AST, ALT and creatinine levels at the end of the experimental period.

Rat group	Normal Control (NC)	Diabetic Control (FR10+STZ)	Mulberry Tea Low (FR10+STZ +MTL)	Mulberry Tea High (FR10+STZ +MTH)	Carnitine (FR10+STZ +CARN)
AST (U/L)	57.86 ± 3.9	59.20 ± 6.3	65.67 ± 14.7	53.20 ± 6.1	52.82 ± 6.4
ALT (U/L)	34.86 ± 6.4	65.20 ± 21.7*	53.83 ± 13.7	62.20 ± 17.8*	59.33 ± 13.8
Creatinine (mmol/L)	0.43 ± 0.1	0.38 ± 0.1	0.39 ± 0.1	0.41 ± 0.1	0.44 ± 0.03

Data are shown as mean ± SD of 7 animals. *$p < 0.05$ vs. NC. (Tukey-Kramer multiple range *post-hoc* test). AST: aspartate transaminase, ALT: alanine transaminase

3.3.11 Glycated protein, total protein, serum albumin and uric acid

Table 9 shows the serum protein profile and uric acid of the different animal groups at the end of the experimental period. No statistical differences were seen among groups for fructosamine. All diabetic groups displayed significantly lower total proteins than the NC group, and in addition, all treatment groups (FR10+STZ+MTL, FR10+STZ+MTH and FR10+STZ+CARN) displayed significantly lower total protein compared to the FR10+STZ group. The treatment groups also displayed significantly lower serum albumin compared to the NC group, but not compared to the FR10+STZ group, which in itself, was not statistically

different from NC. FR10+STZ+MTL and FR10+STZ+CARN displayed significantly lower uric acid levels compared to the NC group, but not compared to the FR10+STZ group.

Table 9: Serum protein profile and uric acid in the animal groups at the end of the experimental period.

Rat group	Normal Control (NC)	Diabetic Control (FR10+STZ)	Mulberry Tea Low (FR10+STZ +MTL)	Mulberry Tea High (FR10+STZ +MTH)	Carnitine (FR10+STZ +CARN)
Fructosamine (µmol/L)	223.7 ± 26.7	242.0 ± 20.4	246.0 ± 17.1	233.0 ± 21.9	238.3 ± 32.6
Total proteins (g/dl)	6.43 ± 0.4	5.58 ± 0.2*	4.88 ± 0.3*#	4.78 ± 0.2*#	4.97 ± 0.2*#
Albumin (g/dl)	2.66 ± 0.3	2.47 ± 0.2	2.28 ± 0.1*	2.25 ± 0.1*	2.29 ± 0.1*
Uric acid (mg/dl)	2.71 ± 1.1	1.90 ± 0.9	1.55 ± 0.4*	1.76 ± 0.2	1.42 ± 0.2*

Data are shown as mean ± SD of 7 animals. *$p < 0.05$ vs. NC, #$p < 0.05$ vs. FR10+STZ. (Tukey-Kramer multiple range *post-hoc* test).

3.4 Discussion

Type 2 diabetes (T2D) is a growing public health threat, with an estimated 171 million people suffering with T2D worldwide in 2000 and this figure is expected to double to 366 million by 2030 (WHO, 2010). Due to the rapidly rising numbers of T2D patients, intensive research on diabetes therapy and prevention has similarly increased. Currently, oral drug therapies for T2D focus on improving insulin secretion and improving insulin sensitivity (Islam and Choi, 2008). Recently there has been an increase in the field of alternative therapies as many drug regimes do not always provide satisfactory glycemic control or prevent later diabetic complications, in addition to the adverse effects produced by many T2D drugs. Natural therapies are thus increasingly sought after by health professionals and patients as an alternative to supplement or replace current regimes to lessen the adverse effects of conventional therapy (Dey *et al.,* 2002).

Tea in traditional Asian medicine is believed to promote both good health and longevity (Yang and Landau, 2000). In recent years, leaves from the mulberry plant have gained much popularity as a tea drink for diabetics in many Asian countries (Butt e*t al.,* 2008). The major anti-diabetic compound in mulberry leaves is a glucose analogue called 1-deoxynojirimycin (DNJ), which inhibits the intestinal enzyme α-glucosidase by binding to the active site in the enzyme (Kojima e*t al.,* 2010). Alpha-glucosidase is considered to be one of the most important enzymes in starch digestion in the small intestine and its inhibition reduces the rate of carbohydrate digestion to prevent extremely elevated blood glucose (BG) levels (Herscovics, 1999). 1-deoxynojirimycin (DNJ) is thus believed to be responsible for the reduction in NFBG and hyperglycemia by reducing the rate of both carbohydrate and lipid absorption (Kojima *et al.,* 2010).

It has been reported that a minimal dose of 6 mg of mulberry DNJ is required to reduce NFBG and decrease insulin secretions (Kimura *et al.,* 2007; Vichasilp *et al.,* 2011). Recently Vichasilp *et al.* (2011) investigated 35 Mulberry tea varieties in Thailand to determine and develop a mulberry tea blend with the optimal DNJ concentration. Several critical findings were established, including determining the selection of leaf area is essential to the level of medicinal potential. Shoots of mulberry leaves contained significantly higher DNJ content than young leaves, which in turn, had significantly higher DNJ content than older mulberry leaves (shoots > young leaves > mature leaves). These findings distinguished better

commercial varieties from those with insufficient DNJ concentrations to elicit any beneficial effect on hyperglycemia.

Moreover, they tested the quality of the tea-making protocol provided by the manufacturers for DNJ extraction at 90 ºC for 300 s. They found an 85% extraction of DNJ from the dry tea material. However, upon modification of the protocol, the optimal tea-making condition was in fact found to be at 98 ºC for 400 s and this increased DNJ extraction to 95%. According to Vichasilp *et al.* (2011), this modification resulted in 0.25 L mulberry tea to be sufficient to provide 6.5 mg DNJ extract and hence sufficient to elicit hypoglycaemic effects. In other words, a 95% DNJ extraction is equivalent of providing 6.5 mg DNJ in 0.25 L of tea, which is only 0.5 mg higher than the minimum required dose to provide any hypoglycaemic effect. This indicates any inferior mulberry leaf selections in tea harvesting and/or coupled with incorrect preparation would most likely provide tea brews containing less than 6.5 mg DNJ and hence little or no anti-diabetic effect is likely to be noted. A major limitation of many mulberry tea brands is the non-specification of an effective dose to reduce NFBG, despite the packaging claims of anti-hyperglycemic effects of the tea (Kimura *et al.,* 2004). Furthermore, Vichasilp *et al.* (2011) acknowledged the fact that for daily home-use it is largely impractical to measure precise tea-brewing temperatures for a specified time period in order to ensure sufficient active ingredients are obtained.

For this reason, despite much literature indicating the hypoglycemic effects (Andallu and Varadacharyulu, 2003; Kong *et al.,* 2008; Miyahara *et al.,* 2004; Naowaboot *et al.,* 2009) of mulberry leaf tea and extracts, it is most likely that the brand of tea used in this experiment might not provide sufficient DNJ to reduce NFBG as seen in Figures 29 and 30 and did not benefit in the glucose tolerance ability as seen in Figure 32. There is, however in fact no record of brewed mulberry leaf tea tested in an animal model for T2D, with most of the studies having dosed the extract itself, or isolating and dosing DNJ into experimental animals to examine the anti-diabetic effects (Kim *et al.,* 1999; Kimura *et al.,* 2007; Kong e*t al.,* 2008; Naowaboot *et al.,* 2009). Although the result is unexpected for our experiment, it brings light to the importance for future studies that will be conducted on teas for anti-diabetic activities. At the same time, consumers need to be aware of highly varying qualities of tea as well as scientists needing to have knowledge of the concentration of active compound within the tea itself.

Having described earlier the two major pathogeneses of T2D as insulin resistance and partial pancreatic β-cell dysfunction characterised by overt hyperglycemia, achieving glycemic control is critical to the management of T2D (Fowler and Deepa, 2010). The failure to do so in this experiment by mulberry leaf tea would result in failure to correct decreased serum insulin as seen in Figure 33, increased liver glycogen as seen in Table 6 as well as other minor symptoms of T2D, namely polyphagia, polydipsia and body weight gain as seen in Figures 25-27.

In line with Kojima *et al.* (2010) who tested mulberry leaf extract in healthy non-diabetic human subjects and found no significant differences in serum triglyceride levels after 12 weeks, our results also showed no significant changes in serum triglycerides compared to the FR10+STZ group, although FR10+STZ+MTL, but not FR10+STZ+MTH, was significantly higher than NC. In addition, in healthy human subjects they found no significant differences in total cholesterol, HDL and LDL after both 6 and 12 weeks. Our results demonstrated no significant differences in total cholesterol in FR10+STZ+MTL compared to NC and FR10+STZ, but significantly lower total cholesterol in FR10+STZ+MTH compared to FR10+STZ+MTL and STZ. This may be due to the higher concentration of DNJ found in FR10+STZ+MTH compared to FR10+STZ+MTL, which has been shown to be effective in decreasing lipid accumulation in rats by β-oxidation, as DNJ is known to increase adiponectin and activate AMP-activated protein-kinase (AMPK) (Tsuduki *et al.,* 2009). This is important as low serum adiponectin is associated with insulin resistance in several animal models (Diez and Iglesias, 2003).

On the other hand, L-carnitine (CARN), unlike mulberry leaf tea, does not primarily function to reduce blood glucose, but rather, acts in the reversal of insulin resistance through the normalization of lipid profiles. Several studies have shown that CARN supplementation improves lipid profiles (Cha, 2008; Mondola *et al.,* 1988; Vacha *et al.,* 1983; Winter *et al.,* 1995) and reduces insulin resistance in humans and experimental animals (Mingrone *et al.,* 1999; Rajasekar *et al.,* 2005; Rajasekar and Anuradha, 2007). The results seen in Table 7 confirm these previous reports, particularly emphasising CARN's effectiveness in normalizing triglyceride levels. In this experiment, the FR10+STZ+CARN group had significantly lower serum triglycerides compared to FR10+STZ, and interestingly, even lower serum triglycerides than the NC group, although this result was non-significant, testifying to its great potential to restore triglyceride levels. Restoration of triglyceride levels is important as these lipids are a significant contributor to hyperlipidemia and related

metabolic disturbances that disrupt insulin signalling resulting in insulin resistance (Barnes and Miner, 2009). This signifies CARN's potential to reverse insulin resistance and/or be a useful preventative agent against the development of insulin resistance. In addition to this, FR10+STZ+CARN significantly reduced total cholesterol compared to FR10+STZ, and similarly to triglyceride concentrations, was also non-significantly lower than the NC, testifying to CARN's suitability as a hypocholesterolemic agent.

Insulin resistance is caused as a result of hyperlipidemia i.e. hypertriglyceridemia, hypercholesterolemia and high levels of circulating free fatty acids (FFAs). Human studies in pregnant women supplemented with 2g CARN/day significantly reduced plasma FFAs, thereby successfully preventing insulin resistance and the development of gestational diabetes (Lohninger *et al.,* 2009). The mechanism by which CARN achieves this is complex (Figure 35). CARN reduces the intramitochondrial acyl-CoA/CoA ratio (Rajesekar and Anuradha, 2007) by promoting fatty acid β-oxidation to acetyl CoA (Figure 35, point 1). The acetyl CoA combines with CARN to form acetylcarnitine by the transfer of acetyl groups catalysed by carnitine-acetyltransferase (CAT) (Figure 35, point 2). This reduction in the acyl-CoA/CoA ratio activates pyruvate dehydrogenase (Calvani e*t al.,* 2000) (Figure 35, point 1), which subsequently decreases intracellular concentrations of citrate (Figure 35, point 2). Because citrate is a key regulator of the glycolytic enzyme phosphofructokinase (PFK), decreases in citrate cause an activation of PFK (Figure 35, point 3), leading to a stimulation of the glycolytic pathway and subsequent glucose metabolism. This essentially clears glucose from the bloodstream leading to a reduction in blood glucose (BG) (Calvani e*t al.,* 2000).

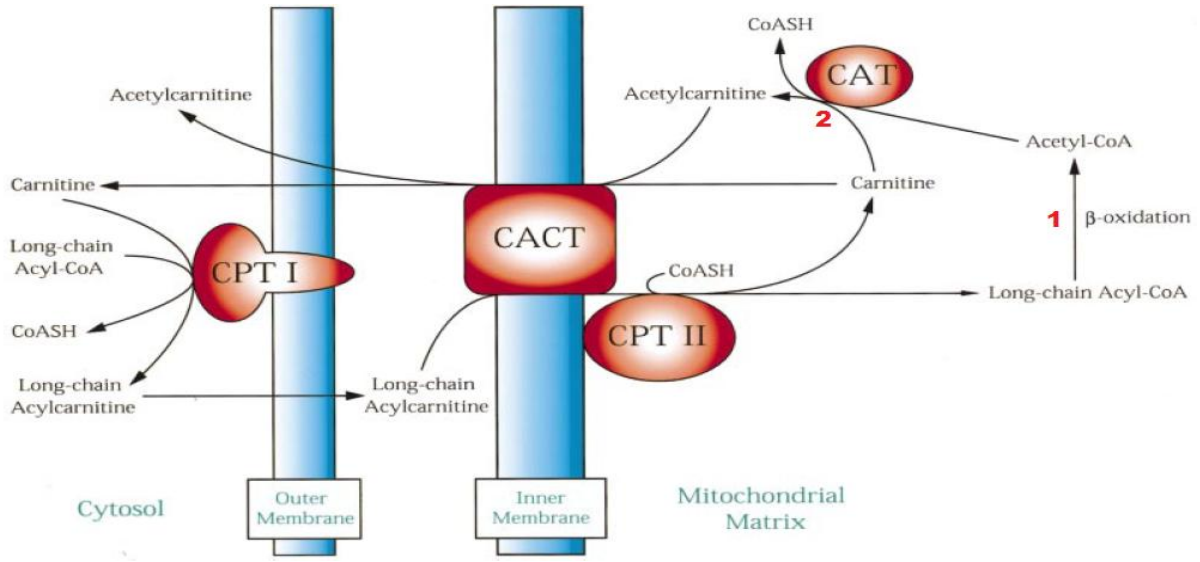

Figure 34: Role of CARN in transport of long-chain fatty acids for β-oxidation (Taken without permission from Vaz and Wanders, 2002).

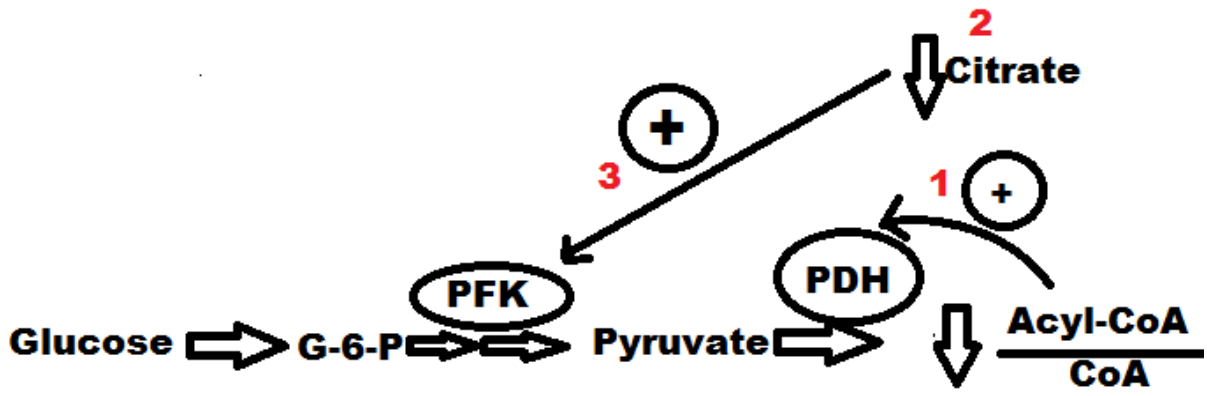

Figure 35: Role of CARN in stimulation of glycolysis and blood glucose reduction (Image self-drawn, adapted and modified without permission from Shulman, 2000).

Research has shown previously of CARN's ability to improve glucose disposal in T2D patients (Capaldo *et al.,* 1991; Mingrone *et al.,* 1999) by decreasing circulating FFAs and improving insulin sensitivity (Capaldo e*t al.,* 1991; Rajasekar e*t al.,* 2005). The results obtained in Figure 31 display no significant improvement in NFBG reduction, although there is a definite non-significant trend toward NFBG reduction, with one animal in this group having fully recovered from overt hyperglycemia within 3-4 weeks (NFBG 421 mg/dl in week 1, 144 mg/dl in week 3, 179 mg/dl in week 4 of the intervention trial) and another animal having reduced NFBG from 600 mg/dl in week 1 to 365 mg/dl in week 4 of the intervention period. These results are intriguing and this leads to much speculation over the success of only 2 of the 7 animals in this group. Supporting the above theory, the animal that recovered fully also displayed the lowest total cholesterol and serum triglycerides of the animals in this group.

In addition to improving lipid profiles and glucose disposal, CARN has previously been found to increase the rate of weight loss in overweight and obese cats (Center *et al.,* 2000). Although in our experiment, at the end of the intervention trial period the FR10+STZ+CARN group had not technically lost weight, the mean weight gain of this group was 10.00g and FR10+STZ+CARN gained the least amount of weight among the animal groups. Taking into account that these are developing and growing rats, one could speculate that in fully developed adult rats this might be translated into weight loss. Furthermore, the FR10+STZ+CARN group did in fact lose weight between weeks 4-6 of the experimental period (Figure 27).

Although hyperglycemia and hypercholesterolemia are the two major contributing factors to the severity of the diabetic condition, it is necessary to analyse other vital biochemical parameters in order to fully assess the anti-diabetic effects of mulberry leaf tea and CARN. In terms of mulberry leaf tea, higher concentrations of DNJ were provided in the brewed tea in the case of the FR10+STZ+MTH group, thus it is prudent to bear in mind that this may increase toxicity due to higher trace elements such as mercury, cadmium and arsenic (Nookabkaew *et al.,* 2006) as well as toxic secondary plant metabolites such as pyrrolizidine alkaloids (Manteiga *et al.,* 1997) commonly found in herbal teas. In order to assess toxicity, liver function enzymes as markers for hepatotoxicity, liver disease and damage were analysed. Alanine transaminase (ALT) and aspartate transaminase (AST) are two common liver enzymes that indicate hepatocyte injury that have leaked into circulation as a result of the disease. Mild but chronic increases of these transaminases are also commonly seen in T2D due to hepatic insulin resistance from elevated levels of circulating free fatty acids, which in fact, are toxic to hepatocytes, presumably due to oxidative stress from lipid peroxidation and the recruitment of inflammatory cells (Harris, 2005). Mulberry leaf tea has been described as hepatoprotective due to its antioxidant potential (El-Beshbishy e*t al.,* 2006). However, in this study, no significant difference was seen between the FR10+STZ+MTL group and the NC group verifying these previous claims, although this may only be relevant to low doses. FR10+STZ+CARN, like FR10+STZ+MTL did not display any significant differences from either the NC or FR10+STZ group, however was non-significantly lower than FR10+STZ, indicating an improved liver function compared to FR10+STZ. CARN is sometimes found to be deficient in T2D, however it is capable of preventing liver injury as seen in Table 8 due to its potent antioxidant activity. Because CARN is involved in transportation of free fatty acids (FFAs) for β-oxidation, less FFAs are available for peroxidation, that otherwise generate oxidative stress and cause liver damage (Flanagan *et al.,* 2010, Rajasekar *et al.,* 2005). In a study conducted by Fallon *et al.* (2008) using a mixture of black (50%), green (20%) and mulberry leaf (30%) tea extracts, ALT but not AST in the 3.0% and 6.0% extract-dosed groups was significantly higher than the control group, indicating possible heptatotoxicity, similar to that seen in FR10+STZ+MTH but not FR10+STZ+MTL in this study compared to the NC group, however neither were statistically different from the FR10+STZ group. Fallon *et al.* (2008) state however that given the mixture of extracts, it is not possible to identify the source of the toxicity, and in addition, the fact that the dosage correlates to quantities that would be extremely high for human consumption levels, which may be similar to that of hepatic necrosis seen in humans with the large

ingestion of tea extracts. In this experiment, it is not possible to conclude whether it is high levels of toxic trace elements in the tea, or whether it is simply an indication of insulin resistance as seen in FR10+STZ (Table 8) and in the previous study FR10 group in Table 4. It is, however, more likely to be an indication of insulin resistance as opposed to hepatotoxicity due to the fact that ALT levels in FR10+STZ+MTH are not significantly different from FR10+STZ.

Besides insulin resistance, serum creatinine is a measure of kidney function as glomerular hyperfiltration is associated with low serum creatinine as well as increased risk for metabolic syndrome and T2D (Hjelmesaeth *et al.,* 2010). No significant differences were noted amongst the groups, despite the model development having significantly lower serum creatinine in STZ and FR10+STZ compared to the NC group in the previous study (Table 4). It is possible that the STZ+FR10 and NC groups in this study did not reveal significant differences due to the three week shorter duration of this study, thus lessening the progression of the disease state.

Uric acid is new risk factor for the prediction of T2D (Dehghan e*t al.,* 2008), with studies showing that moderate hyperglycemia is positively associated with higher uric acid levels, whilst overt hyperglycemia and established T2D is in fact associated with lower uric acid (Yuan *et al.,* 2011). Conversely, high serum uric acid is also a predictor for diabetic complications related to hypertension and cardiovascular disease (Dehghan e*t al.,* 2008). Despite these seemingly conflicting parameters, both mulberry leaf and CARN have been found to possess hypouricemic potential (Yadav and Nade, 2008, Flanagan *et al.,* 2010). A major glycoside of Mulberry plants is mulberroside A, which has been used in Traditional Chinese Medicine for the treatment of gout and hyperuricemia (Wang *et al.,* 2011). Wang *et al.* (2011) found mulberroside A in mulberry plants decreased serum albumin and decreased serum uric acid levels in mice. Amin and Nagy (2009) found administration of 250 mg/kg BW/day CARN significantly decreased serum uric acid and serum urea in high-fat-fed rats after 4 weeks. As a common indicator to insulin resistance, high uric acid also accelerates the development of kidney disease in animals (Cirillo *et al.,* 2006). CARN has shown therapeutic benefit in the prevention of kidney disease and related disorders seen in T2D by counteracting the changes seen in lipid metabolism (Rajasekar *et al.,* 2008). Table 9 confirms the hypouricemic potential of both mulberry leaf tea and FR10+STZ+CARN as both had significantly lower serum uric acid compared to the NC group, and non-significantly lower serum uric acid compared to FR10+STZ. It is thus hard to establish whether these low results

correlate to extreme hyperglycemia and established T2D or as a result of their potent hypouricemic activity. This once again emphasizes the importance of a holistic analysis of all biochemical parameters as opposed to interpreting a single test result.

The fructosamine test is another test that is the result of a non-enzymatic reaction between glucose and amino acids in the serum and is an early glycation product (Cheng-Tzu 2005). Fructosamine can thus be used to predict the concentration of advanced end glycation products (AGES) and is an indicator of glycemic control over a 3 week period (Li and Yang, 2006). Studies indicate that in diabetic animals, fructosamine concentrations increase compared to normal controls (Cheng-Tzu 2005). Low serum fructosamine thus indicates a good glycemic control and in the case of intervention trials, the effectiveness of the treatment regime. However, low fructosamine could also be an indicator of low serum proteins (Browner e*t al.,* 1999) and thus results should not be analysed individually and must be compared in an overall manner to serum albumin and total proteins. For this reason, fructosamine may be falsely low, when combined with low serum albumin and low total proteins. This can be indicative of liver or kidney disorders as well as poorly controlled diabetes. Poorly controlled T2D is associated with altered protein profiles in the body as both reduced circulating insulin and insulin resistance can lead to muscle wasting, denoted by low serum proteins (Castaneda *et al.,* 2000). However, because serum creatinine is directly proportional to muscle mass (Harita 2009), and our results showed no significant differences in serum creatinine, it is unlikely that the cause of low serum proteins is as a result of muscle wasting, and more likely, as a result of poorly controlled diabetes in the case of mulberry leaf tea and FR10+STZ+CARN.

It is difficult to draw solid conclusions of the effectiveness of mulberry leaf tea and CARN based on the serum tests alone. Individually, the test results may lead one to believe that there is good glycemic control, as in the case of the fructosamine tests. However, in conjunction with serum protein profiles and low uric acid, it is more indicative of falsely low results that are actually a result of poorly controlled diabetes.

In summation, mulberry leaf tea did not exhibit any hypoglycemic effects, contrary to many literature reports possibly due to the inferior blend of tea selection and hence was unable to correct minor symptoms of T2D including polyphagia, polydipsia, weight gain and liver glycogen as well as major symptoms including glucose intolerance and insulin deficiency. In accordance with other reports, it did not significantly affect the lipid profile, however did

significantly increase serum liver function enzyme ALT, as well as significantly decreasing serum total protein and albumin and serum uric acid – the combined effect thus indicating poorly controlled diabetes and signifying insulin resistance. In conclusion, mulberry leaf tea did not display any significantly beneficial anti-diabetic effects at least in this experimental condition and further studies involving teas need to be carefully analysed to determine concentrations of active compounds. Attention to manufacturer's tea-brewing protocols also need to be tested and analysed for scientific merit.

On the other hand, although FR10+STZ+CARN had no significant hypoglycaemic effects, it appears to have slowly non-significantly reduced NFBG over the 4 week intervention period, and in addition, significantly reduced some minor symptoms of T2D including polydipsia and resulting in a much less, albeit non-significant, weight gain. Similarly to mulberry leaf tea, it did not significantly affect major symptoms such as glucose intolerance and insulin deficiency, although these results were non-significantly better than mulberry leaf tea and the FR10+STZ group. Coupled with elevated liver enzymes, decreased serum protein and uric acid, CARN is not effective as a single agent to affect glycemic control significantly. CARN, however, was highly effective in normalizing serum lipid profiles, particularly triglycerides, which may aid in the reversal of insulin resistance and prevention of later T2D complications like hypertension and atherosclerosis. Its potent antioxidant activity may also be beneficial in prevention of renal disease (nephropathy) and neuropathy. The success of CARN in normalizing serum lipid profiles indicates its great potential for insulin-resistant-pre-diabetic patients as well as prevention of gestational diabetes, which may indirectly improve glucose disposal in these types of patients but not in chronic diabetic patients. While CARN supplementation alone is not enough to allow for the successful management of T2D, it should be recommended as an additional supplement to conventional drug therapy. The fact that CARN is naturally produced in the body is also advantageous as in diseased states; impairment in endogenous synthesis can be replaced through exogenous dietary consumption without causing serious side effects caused by synthetic drugs. Future studies should be conducted to determine CARN's ability to prevent insulin resistance in healthy subjects or fully reverse insulin resistance in pre-diabetic-insulin-resistant individuals.

Chapter 4 General discussion

The most successful intervention materials should reverse insulin resistance and prevent partial pancreatic β-cell dysfunction. Ideal alternative therapies must exhibit a similar degree of efficacy compared to conventional drug therapy, however without the negative side effects often associated with them (Dey *et al.,* 2002). With over 400 reported traditional medicines for the treatment of T2D, The World Health Organization Expert Committee on diabetes has recognised the great potential of natural plants and functional foods as alternative treatments for T2D and has recommended further investigations to be carried out in support of them (Bailey and Day, 1989). Unfortunately results using traditional plants or functional foods have been difficult to replicate as many studies have utilized non-standardized forms of the compounds. There is thus an urgent need for standardization to allow for successful conclusions to be drawn (Dey e*t al.,* 2002).

In order for this to be achieved, an ideal method of testing these compounds needs to be available to researchers. Studies into disease mechanistics and patterns have exponentially expanded, with the majority of the work performed on animal models (Wall and Shani, 2008). This is because the biochemical pathways in mammals are largely conserved, allowing researchers to predict the outcome in humans. Animal model development thus plays a pivotal role as the foundation for good research in human diseases.

The perfect model for T2D would ideally be inexpensive, easy to develop and simple to maintain along with the identical biochemical profile (hyperglycemia, decreased serum insulin and creatinine, hypercholesterolemia, elevated liver enzymes and liver glycogen) as well as demonstrating the clear pathogeneses of T2D in chronological order of development in humans, insulin resistance and then development of partial pancreatic β-cell dysfunction. Although there are some models available for specified research of a single pathogenesis or a later complication, unfortunately, to date, no single model demonstrates all of the above listed characteristics (Islam and Loots, 2009).

The model developed in this study thoroughly examined all necessary parameters seen in the pathogenesis of T2D and demonstrated in chronological order, the development of insulin resistance and later, partial pancreatic β-cell dysfunction. Biochemical analyses revealed satisfactory results within the parameters for the profile of human T2D. No tests however were performed to assess its suitability for later diabetic complications such as nephropathy,

neuropathy, retinopathy and cardiomyopathy, thus one cannot speculate on its use for chronic diabetic studies.

For acute studies however, such as studies on the anti-diabetic effects of plant and functional foods, it was therefore necessary to validate the newly developed model through a therapeutic intervention trial. The results of the intervention trial demonstrated both suitability of this model of further and future acute studies as well as concurrently adding critical research to the body of knowledge on functional foods and plant therapies.

In conclusion, the model developed may be a useful, novel, non-genetic alternative model for T2D characterized by all major pathogeneses. The model additionally provided highly satisfactory results when validated through an intervention trial. The intervention trial revealed that mulberry leaf tea and CARN are not suitable as a substitute therapy for T2D, but may in some cases, be beneficial in combination with conventional drug therapy. Future studies should be conducted on these intervention materials to determine the most effective dosage in treating T2D symptoms as well as assess their effect in other areas related to this disease. Future studies should also most definitely be performed on this model to assess and determine its suitability for more broad scale studies, particularly those involving chronic diabetic complications. The biggest advantage that already exists for chronic studies is the cheap and simple development and well as low maintenance costs when using this model, which, for developing countries, is extremely useful and promising.

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Dear Dr. Islam,

We are enclosing your manuscript entitled "Fructose-fed streptozotocin-injected rat: an alternative model for type 2 diabetes" (no. 4898) with editorial remarks. Please read carefully all comments in the text.

Would you please additionally consider quoting in your publication the papers published in "Pharmacological Reports" in the years 2010-2011, e.g. such items as: (1/ Łabuzek K, Liber S, Gabryel B, Okopień B: Metformin has adenosine-monophosphate activated protein kinase (AMPK)-independent effects on LPS-stimulated rat primary microglial cultures. Pharmacol Rep, 2010, 62, 827-848. 2/ Cai Z, Zhao Y, Yao S, Zhao B: Increases in beta-amyloid protein in the hippocampus caused by diabetic metabolic disorder are blocked by minocycline through inhibition of NF-kappaB pathway activation. Pharmacol Rep, 2011, 63, 381-391).

After exact correction, new version of your article ought to be sent back **until January 27th** (named as: **4898_last**).
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>>> "Hans-Georg Joost" <joost@dife.de> 2/6/2012 12:07 PM >>>
Dear colleagues,

This note is to inform you that all chapters of our volume Animal Models in Diabetes Research have been finally sent to the publisher. Therefore, I think that the contributions can be considered 'in press' now. We missed the deadline by 2 months which does not seem too bad to me. Those of you who sent in your chapters early: Please accept my apologies for the delay.

I expect that you will eventually receive proofs from the copy editor and also - if necessary - requests as to missing or unclear items which escaped my editing.

I wish to take the opportunity and thank you for your excellent work. I believe that your contributions have produced an outstanding volume which will be an important source and reference in experimental diabetes research.

With best personal regards,

Hans

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