



Effects of Commercially Available Non-Nutritive Sweeteners in an Experimentally Induced Rat Model of Type 2 Diabetes

By

Siphiwe Ndumiso Dlamini

208524703

A dissertation submitted in partial fulfillment of the requirements for the degree
of Master of Science in Biochemistry

College of Agriculture, Engineering and Science

School of Life Sciences

Discipline of Biochemistry

Supervisor: Dr Md. Shahidul Islam

December 2014

PREFACE

The experimental work presented in this thesis was carried out in the Department of Biochemistry; School of Life Sciences; College of Agriculture, Engineering and Science at the University of KwaZulu-Natal, Westville Campus; from January 2013 to November 2014.

The thesis describes original work of the author (except where specifically acknowledged) and has not been previously accepted for any degree and is not being currently considered for any other degree at any other university.

Signed.....

Date.....

DECLARATION 1 - PLAGIARISM

I, Siphiwe Ndumiso Dlamini, student number: 208524703, declare that:

- I. The research described in this thesis is my own original work, except where indicated in the text.
- II. This thesis has not been submitted for any examination or degree at any other institution.
- III. This thesis does not contain any other persons' information, pictures, graphs, data, etc., except where specifically acknowledged.
- IV. This thesis does not contain data, graphics, tables or other information copied and pasted from the internet, unless where specifically acknowledged as being sourced from a specific website.

Signed.....

Date.....

DECLARATION 2 – PUBLICATIONS

This declaration gives details of publications that form part of research presented in this thesis. These include publications still in preparation, submitted, *in press* and published and describes the contributions of each author to the experimental work as well as preparation of each manuscript.

Publication 1

Dlamini SN, Mbambo NP, Islam MS. Commercially available sucralose-containing sweetener worsens lipid profile, inflammatory biomarkers and organ histopathology in normal and type 2 diabetes model of rats. **(In preparation)**

Publication 2

Dlamini SN, Mbambo NP, Islam MS. Commercially available saccharin-containing sweeteners may not be suitable for patients with type 2 diabetes. **(In preparation)**

Publication 3

Dlamini SN, Mbambo NP, Islam MS. Effects of commercially available aspartame-containing sweetener in an experimentally induced rat model of type 2 diabetes. **Basic and Clinical Pharmacology and Toxicology** (Revision requested by the journal).

Publication 4

Dlamini SN, Mbambo NP, Islam MS. Effects of commercially available cyclamate-containing sweetener in an experimentally induced rat model of type 2 diabetes. **(In preparation)**

Publication 5

Dlamini SN, Mbambo NP, Islam MS. Commercially available stevia-based sweetener, anti-diabetic or toxic: An *ad libitum* feeding study in a type 2 diabetes model of rats. (Submitted to **Food and Function Journal**)

All experimental work was done by Siphiwe N Dlamini and Nondumiso P Mbambo, and assisted by Dr MS Islam where necessary. All manuscripts were written by Siphiwe N Dlamini and revised and edited by Dr MS Islam.

Signed by student:

Signed by supervisor:

DECLARATION 3 – PRESENTATIONS

This declaration gives details of publications that form part of research presented in this thesis.

Presentation 1:

Effects of commercially available sugar substitutes in an experimentally induced rat model of type 2 diabetes. The 49th Congress of the Society for Endocrinology, Metabolism and Diabetes of South Africa, 11-13 April, 2014, Elangeni Hotel, Durban, South Africa.

Awarded: *Registration grant by the Society for Endocrinology, Metabolism and Diabetes of South Africa to present this work in the above mentioned congress.*

Presentation 2:

Effects of commercially available sugar substitutes in an experimentally induced rat model of type 2 diabetes. University of KwaZulu-Natal (UKZN) School of Life Sciences 2nd Annual Research day, 26 May, 2014, Westville Campus, Durban, South Africa.

Signed by student:

Signed by supervisor:

ACKNOWLEDGEMENTS

First and foremost, I would like to thank God for the grace that he granted me to pursue and finish my work. “But by the grace of God I am what I am, and his grace to me was not without effect. No, I worked harder than all of them- yet not I, but the grace of God that what was with me.” 1 Corinthians 15:10.

I would also like to thank the following people who contributed significantly in my research:

A special thanks to my supervisor Dr MS Islam for his constant support and supervision throughout the whole period of my Masters study. I’m grateful for his contribution in pushing me to the edges of my limits and beyond. I would have never achieved my MSc degree without his valuable guidance, knowledgeable skills and expertise and his inclination to assist me even when he didn’t have to. Through my supervisor I have learnt to become a real scientist, as he has considerably contributed in improving my skills in research, scientific writing, presentation and applying for research funding and scholarships. Thus I am more than grateful to have had a supervisor such as Dr Islam, and I couldn’t have chosen anyone better.

I would also like to thank the members from my lab, especially Nondumiso Mbambo for her constant and much appreciated support in conducting the experimental work presented in this thesis. She was always there to assist as my research partner. Through her knowledge I have learnt to think differently and be more organized especially in terms of using Microsoft excel and designing experimental research. Thus I sincerely appreciate her partnership in conducting my research.

Thank you to the Biomedical Resource Unit (BRU) technical staff members, especially Ritta, Linda, David and Dennis for their assistance in training me in handling and working with animals, for taking care of the animals, for maintaining hygiene in the animal room and for their assistance whenever needed. I would also like to thank Mrs Shoohana Singh from the Department of Physiology for her training in conducting histopathology, for allowing me to do some of my research in her lab and for her constant support and patience, and everything else she did for me.

To my friends and family:

Thank you, especially to my parents for their moral and financial support, and motivation, without their existence I would have never achieved much in life. They have always been there for me for as long as I can remember and have never disappointed me or let me down in any way. I thank God for giving me such good parents. Thank you to my friends and family from church, for their prayers and motivation to continue doing my school work. I'm also appreciative for the moral and financial support I received from my siblings and my cousins (especially Sindi Dlamini and Sihle Mngadi), and my Aunt.

Finally, I highly acknowledge the scholarship I received during my MSc study from the South African Medical Research Council (SAMRC). Moreover, this study was also supported by the Nutrition Research Grant from South African Sugar Association (SASA, Project 233) and Competitive Research Grant from the University of KwaZulu-Natal, Durban; Grant Support for Young Men and Women researchers from the National Research Foundation (NRF), Pretoria, South Africa. Thus, these funding supports were highly appreciated.

ABSTRACT

Non-nutritive sweeteners (NNSs) are becoming more popular as sugar substitutes for diabetic patients. Most studies have reported conflicting results and have used NNSs in their pure form; and not in the form consumed by people. Thus, the present study primarily aimed at investigating the effects of commercially available NNSs in an experimentally induced rat model of type 2 diabetes (T2D). The study also assessed the toxicological effects of these NNSs in normal rats. Seven-week-old male Sprague-Dawley rats were randomly divided into eleven groups, as follows: Normal Control (NC), Diabetic Control (DBC), Diabetic Aspartame (DASP), Diabetic Sucralose (DSCL), Diabetic Cyclamate (DCLM), Diabetic Saccharin (DSAC), Diabetic Stevia (DSTV), Toxicological Aspartame (TASP), Toxicological Sucralose (TSCL), Toxicological Cyclamate (TCLM) and Toxicological Saccharin (TSAC). T2D was induced experimentally in the DBC, DASP, DSCL, DCLM, DSAC and DSTV groups only. In order to induce the two major characteristics of T2D, the rats were fed 10 % fructose solutions *ad libitum* for two weeks to induce insulin resistance and then intraperitoneally injected with 40 mg/kg BW streptozotocin (STZ). Rats with three hour fasting blood glucose (3h-FBG) concentrations ≥ 300 mg/dl were considered as diabetic. During the 13 week experimental period, control groups were administered with normal drinking water; when treated diabetic (DASP, DSCL, DCLM, DSAC and DSTV) and toxicological (TASP, TSCL, TCLM and TSAC) groups were receiving their respective NNS solutions, *ad libitum*, at concentrations equivalent to the sweetness of 10% and 20% sucrose solution, respectively. Although consumption of commercially available NNSs to normal rats reduced serum lipids and fructosamine, the rats presented tissue damage concomitant with increased serum inflammatory biomarkers. In the diabetic groups, consumption of NNSs influenced serum lipid profile and worsened tissue injury which was also confirmed by increased inflammatory biomarkers. The results of this study indicated that consumption of commercially available NNSs can profoundly influence diabetes related parameters and its related complications in both normal and type 2 diabetic rats. Thus it is recommended that individuals using NNSs should limit their daily intake.

CONTENTS

	Page No.
ABBREVIATIONS	i
LIST OF TABLES.....	iii
LIST OF FIGURES	iv

CHAPTER ONE

General introduction

1.1 Literature review	2
1.1.1 Diabetes overview.....	2
1.1.2 Type 1 diabetes	3
1.1.3 Type 2 diabetes	3
1.1.4 Pathogenesis of type 2 diabetes	4
Insulin resistance.....	4
Pancreatic β -cell dysfunction	6
1.1.5 Prevalence of type 2 diabetes.....	7
1.1.6 Management of type 2 diabetes	7
Dietary management and type 2 diabetes	8
Physical activity and type 2 diabetes	9
Medical therapy for type 2 diabetes	9
1.1.7 Commonly Used Sweeteners	12
Natural sweeteners	12
1.1.8 Artificial sweeteners	20
Saccharin.....	21
Aspartame	23
Sucralose.....	25
Cyclamate	27

1.2 Rationale of the study	28
1.3 Aims of the study	29
1.4 Objectives	29

CHAPTER TWO

Materials and methods

2.1 Rats, housing and feeding	30
2.2 Induction and confirmation of diabetes	31
2.3 Measurement of food and fluid intake, and body weight gain.....	32
2.4 Non-fasting blood glucose and the oral glucose tolerance test	32
2.5 Collection of blood and tissue samples.....	33
2.6 Serum analysis	33
2.6.1 Serum lipid profile	33
2.6.2 Enzymes in the serum	35
2.6.3 Serum fructosamine, creatinine and uric acid.....	37
2.6.4 Serum insulin	38
Principle	38
Procedure	38
2.7 Histopathological examination of the tissue samples	39
2.7.1 Sample tissue processing and sectioning	39
2.7.2 Slide staining and viewing	39
2.8 Statistical Analysis.....	39

CHAPTER THREE

Commercially available sucralose-containing sweetener worsens lipid profile, inflammatory biomarkers and organ histopathology in normal and type 2 diabetes model of rats

3.1 Abstract.....	42
3.2 Introduction.....	42
3.3 Materials and methods	44

3.4 Results.....	44
3.4.1 Calculation of the human equivalent dose of sucralose	44
3.4.2 Food and fluid intake, 3h-FBG and OGTT	45
3.4.3 Serum lipid profile	47
3.4.3 Serum ALP and liver function enzymes	48
3.4.4 Serum insulin, fructosamine, LDH, CK-MB, uric acid and creatinine.....	49
3.4.5 Histopathological findings	51
3.5 Discussion.....	53
3.6 Conclusions.....	56
3.7 References.....	56

CHAPTER FOUR

Commercially available saccharin-containing sweeteners may not be suitable for patients with type 2 diabetes

4.1 Abstract.....	58
4.2 Introduction.....	58
4.3 Materials and methods	60
4.4 Results.....	60
4.4.1 Daily food and fluid intake, and body weight gain.....	60
4.4.2 Non-fasting blood glucose and oral glucose tolerance test.....	61
4.4.3 Serum lipid profile	64
4.4.4 Serum insulin, fructosamine, ALP, ALT, AST, LDH, CK-MB, uric acid, and creatinine	65
4.4.5 Histopathological findings	66
4.5 Discussion.....	68
4.6 Conclusions.....	70
4.7 References.....	70

CHAPTER FIVE

Effects of commercially available aspartame-containing sweetener in an experimentally induced rat model of type 2 diabetes

5.1 Abstract	72
5.2 Introduction.....	73
5.3 Materials and methods	74
5.4 Results.....	74
5.4.1 Food and fluid intake, and body weight.....	74
5.4.2 Weekly 3h-FBG and OGTT	75
5.4.3 Serum lipid profile	77
5.4.4 Liver function enzymes in the serum.....	78
5.4.5 Serum insulin, fructosamine, LDH, CK-MB and creatinine.....	79
5.4.6 Histopathological findings	81
5.5 Discussion.....	83
5.6 Conclusions.....	86
5.7 References.....	86

CHAPTER SIX

Effects of commercially available cyclamate containing sweetener in an experimentally induced rat model of type 2 diabetes

6.1 Abstract	88
6.2 Introduction.....	88
6.3 Materials and methods	90
6.4 Results.....	90
6.4.1 Daily food and fluid intake, and body weight gain.....	90
6.4.2 Weekly non-fasting blood glucose and oral glucose tolerance test	91
6.4.3 Serum lipid profile and serum enzymes.....	93
6.4.4 Serum insulin, fructosamine, uric acid and creatinine concentrations.....	96
6.4.5 Histopathological findings	96

6.5 Discussion.....	99
6.6 Conclusion	101
6.7 References.....	101

CHAPTER SEVEN

Commercially available stevia-based sweetener, anti-diabetic or toxic: An *ad libitum* feeding study in a type 2 diabetes model of rats

7.1 Abstract.....	103
7.2 Introduction.....	103
7.3 Materials and methods	105
7.4 Results.....	105
7.4.1 Calculation of the human equivalent dose of stevia glycosides.....	105
7.4.2 Food and fluid intake, and weekly body weight change	106
7.4.3 Weekly non-fasting blood glucose and oral glucose tolerant test.....	108
7.4.4 Serum insulin, fructosamine, uric acid and creatinine concentrations.....	111
7.4.5 Serum lipid profile and serum enzyme concentrations	112
7.4.6 Histopathological examination of organ tissues	114
7.5 Discussion.....	116
7.6 Conclusion	118
7.7 References.....	119

CHAPTER EIGHT

General discussion and conclusion

8.1 General discussion	120
8.2 General conclusion.....	122
References.....	126

ABBREVIATIONS

ADA	: American diabetes association
ADI	: Acceptable daily intake
ALP	: Alkaline phosphatase
ALT	: Alanine transaminase
AST	: Aspartate transaminase
BRU	: Biomedical resource unit
BW	: Body weight
CK-MB	: Creatine kinase
DASP	: Diabetic aspartame
DBC	: Diabetic control
DCLM	: Diabetic cyclamate
DM	: Diabetes mellitus
DSAC	: Diabetic saccharin
DSCL	: Diabetic sucralose
DSTV	: Diabetic stevia
ELISA	: Enzyme-linked immunosorbent assay
FBG	: Fasting blood glucose
FDA	: Food and Drug Administration
GLP-1	: Glucagon-like peptide 1
HDL	: High-density lipoproteins
IDF	: International diabetes federation
LDL	: Low-density lipoproteins
NC	: Normal control
3h-FBG	: 3 hour fasting blood glucose
NNS	: Non-nutritive sweetener

OGTT	: Oral glucose tolerance test
STZ	: Streptozotocin
T1D	: Type 1 diabetes
T2D	: Type 2 diabetes
TASP	: Toxicological aspartame
TCLM	: Toxicological cyclamate
TSAC	: Toxicological saccharin
TSCL	: Toxicological sucrose

LIST OF TABLES

	Page No.
Table 2.1 Grouping of animals and NNS solutions	31
Table 3.1 Calculation of the human equivalent dosages and actual consumption of sucralose by normal and diabetic rats via drinking water	45
Table 3.2 Serum LDH, uric acid, fructosamine, CK-MB, creatinine and insulin at the end of the experimental period in sucralose consuming rats	50
Table 4.1 Serum insulin, fructosamine, ALP, ALT, AST, LDH, CK-MB, uric acid, and creatinine at the end of the experimental period in saccharin consuming rats	62
Table 5.1 Serum insulin, fructosamine, LDH, CK-MB, uric acid, and creatinine concentrations at the end of the 13-week experimental period in aspartame consuming rats	91
Table 6.1 Serum lipid profile and serum enzymes at the end of the experimental period in cyclamate consuming rats	95
Table 7.1 Calculation of the human equivalent dosages and actual consumption of stevia by the rats in drinking water in stevia consuming rats	106
Table 7.2 Serum enzymes at the end of the experimental period in stevia consuming rats	113
Table 8.1 Comparing the effects of each NNS in the diabetes and toxicity studies	123

LIST OF FIGURES

	Page No.
Figure 1.1 Pathophysiology of visceral fat, insulin resistance and type 2 diabetes	5
Figure 1.2 Hyperglycemia-induced β -cell dysfunction, insulin resistance, and T2D	6
Figure 1.3 Chemical structures of commonly used sugar alcohols	14
Figure 3.1 Mean food and fluid intake and body weight gain during the entire experimental period (Sucratose study)	45
Figure 3.2 Mean weekly non-fasting blood glucose in different rats groups over the 13 week experimental period (Sucratose study)	46
Figure 3.3 Oral glucose tolerance test (OGTT) in different animal groups at week 12 of the 13 week experimental period (Sucratose study)	46
Figure 3.4 Serum lipid profile in the different animal groups at the end of the experimental period (Sucratose study)	48
Figure 3.5 Concentrations of alkaline phosphates and liver function enzymes in the serum of different animal groups at the end of the experimental period (Sucratose study)	49
Figure 3.6 Histological examinations of the brain, liver, heart, kidney, and pancreas of different animal groups at the end of the experimental period (Sucratose study)	52
Figure 4.1 Mean food and fluid intake and body weight gain during the entire experimental period (Saccharin study)	60
Figure 4.2 Mean weekly non-fasting blood glucose in different rats groups over the 13 week experimental period (Saccharin study)	61
Figure 4.3 Oral glucose tolerance test (OGTT) in different animal groups at week 12 of the 13 week experimental period (Saccharin study)	63

Figure 4.4	Serum lipid profile in the different animal groups at the end of the experimental period (Saccharin study)	63
Figure 4.5	Histological examinations of the brain, liver, heart, kidney and pancreas of different animal groups at the end of the experimental period	66
Figure 5.1	Mean food and fluid intake and body weight gain during the entire experimental period (Aspartame study)	75
Figure 5.2	Mean weekly non-fasting blood glucose in different rats groups over the 13 week experimental period (Aspartame study)	76
Figure 5.3	Calculated area under the curve (AUC) for weekly non-fasting blood glucose in different rats groups over the 13 week experimental period (Aspartame study)	76
Figure 5.4	Oral glucose tolerance test (OGTT) in different animal groups at week 12 of the 13-week experimental period (Aspartame study)	77
Figure 5.5	Serum lipid profile in the different animal groups at the end of the experimental period(Aspartame study)	78
Figure 5.6	Concentrations of alkaline phosphates and liver function enzymes in the serum of different animal groups at the end of the experimental period (Aspartame study)	79
Figure 5.7	Histological examinations of the brain, liver, heart, kidney and pancreas of different animal groups at the end of the experimental period (Aspartame study)	82
Figure 6.1	Mean food and fluid intake and body weight gain during the entire experimental period (Cyclamate study)	91
Figure 6.2	Weekly non-fasting blood glucose in different rats groups over the 13 week experimental period (Cyclamate study)	92

Figure 6.3	Oral glucose tolerance test (OGTT) in different animal groups at week 12 of the 13 week experimental period (Cyclamate study)	93
Figure 6.4	Serum lipid profile in the different animal groups at the end of the experimental period (Cyclamate study)	94
Figure 6.5	Serum insulin, fructosamine, uric acid and creatinine concentrations of different animal groups at the end of the experimental period (Cyclamate study)	96
Figure 6.6	Histological examinations of the brain, liver, heart, kidney and pancreas of different animal groups at the end of the experimental period (Cyclamate study)	98
Figure 7.1	Mean food and fluid intake over the 13 week experimental period (Stevia study)	107
Figure 7.2	Mean weekly body weight during the entire experimental period (Stevia study)	108
Figure 7.3	Mean weekly non-fasting blood glucose in different rats groups over the 13 week experimental period (Stevia study)	109
Figure 7.4	Oral glucose tolerance test (OGTT) in different animal groups at week 12 of the 13 week experimental period (Stevia study)	110
Figure 7.5	Serum insulin, fructosamine, uric acid and creatinine concentrations of different animal groups at the end of the experimental period (Stevia study)	111
Figure 7.6	Serum lipid profile in different animal groups at the end of the 13 week experimental period (Stevia study)	112
Figure 7.7	Histological examinations of the brain, liver, heart, kidney and pancreas of different animal groups at the end of the experimental period (Stevia study)	115

CHAPTER ONE

General introduction

Diabetes is a global public health problem and affects more than 382 million people worldwide (International Diabetes Federation: IDF, 2013). It can be defined as a metabolic disorder characterized by chronic hyperglycemia; which is caused by impaired insulin action, secretion or both. This disease is classified into two major types; type 1- and type 2-diabetes, when type 2 accounts for 90-95% of all diabetes cases. Although the pathogenesis of type-2 Diabetes (T2D) is not fully understood, this form of diabetes is characterized by progressive decline in insulin action (insulin resistance) and inability of the β -cells to compensate for insulin resistance (β -cells dysfunction) (American Diabetes Association: ADA, 2013). Additionally, excessive hepatic gluconeogenesis also contributes to hyperglycemia as well as to diabetes (Rendell, 2004).

Even though T2D is not well managed in the world including South Africa, there are specific guidelines for its management. According to the 2012 Society for Endocrinology, Metabolism and Diabetes of South Africa (SEMDSA) guidelines for management of T2D, patients with this form of diabetes should implement healthy lifestyle changes; such as reducing calorie intake in their diet and increasing physical activity (SEMDSA, 2012). Moreover, high dietary intake of sucrose and fructose, especially in the form of liquid, has been consistently linked to increase in energy intake and body weight gain, risk of developing visceral adiposity, insulin resistance, dyslipidemia and T2D (Schulze et al., 2004; Dhingra et al., 2007; Stanhope et al., 2009; Hu and Malik, 2010). On the other hand, overweight, obese and diabetic people always try to reduce their total calorie intake by consuming non-nutritive sweeteners (NNSs) instead of refined sugar or sucrose. Thus, NNSs have become highly popular sugar substitutes for individuals with above-mentioned disorders. Additionally, significantly higher sweetness and no or very lower calorie content compared to sucrose has made these sweeteners more popular to the diabetic patients. Several studies have been conducted to assess whether NNSs possess health risks as food additives or not (Benton, 2005; Whitehouse et al., 2008; Brown et al.,

2010; Yang, 2010; Brown and Rother, 2012). However, the reports of these studies are largely conflicting, with conclusions ranging from safe at all concentrations to unsafe at any concentration.

Since NNSs are calorie-free, they are not expected to promote weight gain when consumed as food additives. A number of clinical studies and some animal trials have supported this statement and have reported that NNS do not influence food intake and body weight gain (Baird et al., 2000; Hunty et al., 2006; Park et al., 2010;). On the other hand, some animal studies have reported that intake of NNSs can induce body weight gain by increasing food or energy intake and/or by decreasing energy expenditure (Collison et al., 2012^a ; Feijo et al., 2013; Swithers et al., 2013). Moreover, there is no consistent evidence that NNSs affect the glycemic responses in healthy humans (Anton et al., 2010; Ford et al., 2011; Bryant et al., 2014). In contradiction to these reports, several animal studies have shown that consumption of NNSs may impair glucose homeostasis and thus influence other diabetes-related parameters (Swithers and Davidson, 2008; Collison et al., 2012^b). Thus whether consumption of NNSs is effective in the management of diabetes or not, remains highly debatable.

Moreover, majority of the studies have used NNSs in their pure form and not in the form that is consumed by people. Recent studies have reported that NNSs may have synergistic or additive effects when used with other food additives (Lau et al., 2006; Collison et al., 2012^b). Since NNSs are usually used in combination to obtain a better sweetness profile, we hypothesized that commercially available NNSs may have different effects to what has been previously reported.

1.1 Literature review

1.1.1 Diabetes overview

Diabetes is one of the most common metabolic disorders and is characterized by chronic hyperglycemia; which is caused by impaired insulin action, secretion or both. Chronic hyperglycemia is marked by a number of classical symptoms, viz.: polydipsia, polyphagia, polyuria, weight loss and sometimes impaired vision (ADA, 2013). Moreover, uncontrolled hyperglycemia can ultimately lead to the development of serious long term complications which include: diabetic nephropathy with

potential of renal failure; diabetic retinopathy which can cause permanent loss of vision; diabetic neuropathy which can lead to even limb amputation; and autonomic neuropathy with the risks of cardiovascular symptoms and sexual dysfunction (Fowler, 2008; Forbes and Cooper, 2013). Furthermore, diabetic patients often have abnormalities in lipid metabolism and an increased risk of developing diseases such as hypertension, and cerebrovascular and cardiovascular diseases (Turner et al., 1998).

1.1.2 Type 1 diabetes

Type 1 Diabetes (T1D) results from the autoimmune destruction of β -cells which leads to insulin deficiency and ultimately hyperglycemia (Kuzuya et al., 2002). This class of diabetes accounts for only 5-10% of all diabetes cases and is also known as insulin dependent or juvenile-onset diabetes (ADA, 2013). Destruction of β -cells results into T1D regardless of the causal factor, and this usually progresses to absolute insulin deficiency, thus patients with this form of diabetes require insulin for survival (Van Belle et al., 2011). The most common form of T1D (85-90% of patients) is caused by a cellular-mediated autoimmune destruction of the β -cell thus a number of autoantibodies are detected during the initial fasting hyperglycemia in majority of T1D patients which include; autoantibodies to insulin and autoantibodies to tyrosine phosphatases IA-2 and IA-2 β (Plagnol et al., 2011).

1.1.3 Type 2 diabetes

Type 2 diabetes (T2D) is characterized by progressive decline in insulin action (insulin resistance) and inability of the β -cells to compensate for insulin resistance (β -cells dysfunction). This form of diabetes accounts for 90-95% of all diabetes cases, and was previously referred to as non-insulin dependent or adult-onset DM (ADA, 2013). Although the etiology of T2D is not fully understood, autoimmune destruction of β -cells does not occur in this type of diabetes (Kuzuya et al., 2002). The majority of patients with this form of diabetes are often overweight or obese, and have increased amount of body fat (particularly abdominal) which causes insulin resistance at least to a certain extent. In T2D, hyperglycemia develops progressively and thus this form of diabetes can go

undiagnosed for years, especially at initial stages where hyperglycemia is not severe enough to produce the classical symptoms of T2D (Meigs et al., 2003).

Other common forms of diabetes

Other common types of diabetes include gestational diabetes and maturity onset diabetes of the young (MODY). Gestational diabetes can be defined as a condition in which a woman without previously diagnosed diabetes exhibits hyperglycemia during pregnancy (ADA, 2013). This form of diabetes may not produce most of the classical symptoms of diabetes and commonly detected by screening during pregnancy (SEMDSA, 2012). On the other hand, MODY refers to any of the hereditary types of diabetes which are caused mutations in an autosomal dominant gene ultimately disrupting the production of insulin (ADA, 2015).

1.1.4 Pathogenesis of type 2 diabetes

Insulin resistance

One of the major characteristics of T2D is insulin resistance, which is defined as progressive decline in insulin action. The physiological functions of insulin include enhancing uptake of glucose by the skeletal muscle and adipose tissue, suppressing glycogenolysis and gluconeogenesis in the liver, and inhibiting lipolysis (Griffith et al., 2010). Thus, insulin resistance is marked by increased circulating glucose, insulin and lipids (Samuel and Shulman, 2012). According to Griffith et al. (2010), the mechanisms by which these changes occur in the serum remains to be fully elucidated. Nonetheless development of insulin resistance has been strongly associated with increased visceral adiposity. Further, animal and clinical studies have shown that free fatty acids and adipokines derived from visceral adipose tissue (VAT) play a major role in the pathogenesis of insulin resistance (Griffith et al., 2010; Samuel and Shulman, 2012).

According to Griffith et al. (2010), visceral fat plays a significant role in the development of insulin resistance and ultimately T2D (Figure 1.1). Although a study by Saiki et al. (2004) revealed that hyperinsulinemia enhances the development of visceral fat, the mechanism by which this occurs, remains unclear. Moreover, the mechanism by which visceral fat causes insulin resistance remains to

be fully elucidated (Griffith et al., 2010). However, it has been reported that increased visceral fat leads to increased free fatty acids (FFAs) in circulation. Since FFAs from the blood drain directly into the liver, elevated production of FFAs lead to increased flux in the liver (Jensen, 2006). The increased FFAs in the liver then increase gluconeogenesis and this diminishes the ability of insulin to inhibit the hepatic production of glucose (Griffith et al., 2010).

In addition, visceral adipose tissue (VAT) serves as an endocrine organ that produces adipokines, signalling proteins that influence lipid and glucose metabolism (Weyer et al., 1999). As VAT increases, the expression of adipokines such as interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), 11 β hydroxysteroid dehydrogenase (11 β -HSD-1), cannabinoid (CB)-1 receptors and plasmogen activator inhibitor-1 (PAI-1) is increased, whilst the expression of adiponectin is decreased (Jensen, 2006). These changes in the genetic expression of the above-listed adipokines negatively affect lipid and glucose metabolism, and ultimately promote insulin resistance (Griffith et al., 2010).

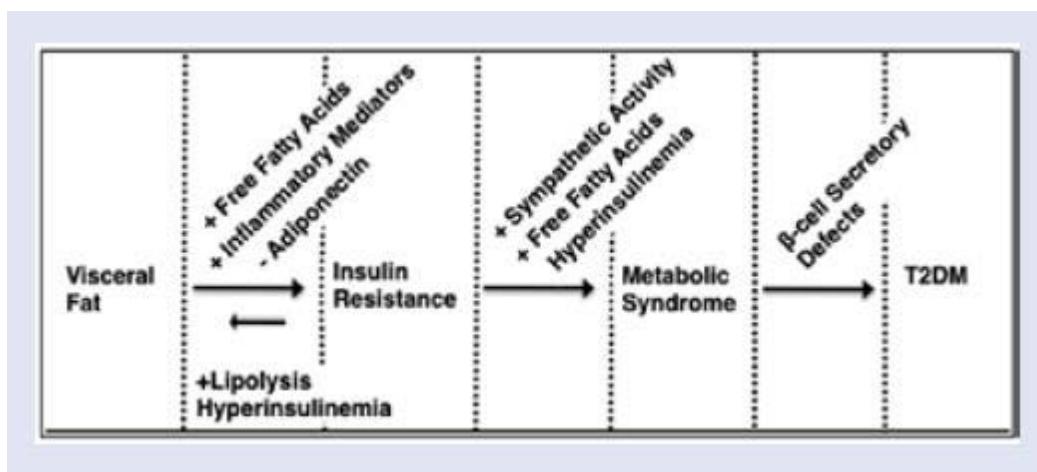


Figure 1.1: Pathophysiology of visceral fat, insulin resistance and type 2 diabetes (copied without permission from Griffith et al., 2010)

β-cell dysfunction

The other major cause of T2D is β-cell dysfunction, which is defined by the inability of the β-cells to secrete sufficient insulin to meet insulin demand. However, the association between insulin resistance and β-cell dysfunction remains to be fully elucidated. As shown in Figure 1.2, hyperglycemia can induce both characteristics of T2D, but β-cell dysfunction is more critical than insulin resistance (Cerf, 2013). In support, individuals with insulin resistance (e.g. during obesity) can still maintain normal blood glucose concentrations, as long as β-cells are able to compensate by secreting more insulin. Figure 1.2 also depicts that as both insulin resistance and β-cell dysfunction are exacerbated, hyperglycemia is worsened ultimately leading to the development of T2D (Figure 1.2).

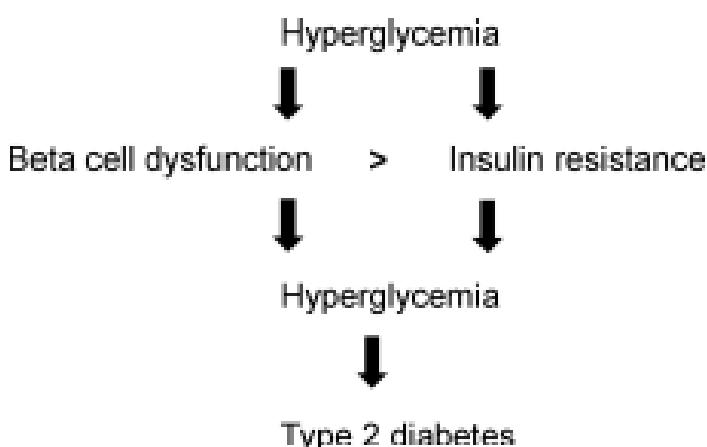


Figure 1.2: Hyperglycemia-induced β-cell dysfunction, insulin resistance, and T2D (copied without permission from Cerf, 2013)

Interestingly, studies have demonstrated that risk factors for T2D in healthy individuals act through β-cell dysfunction rather than insulin resistance, suggesting that β-cell function and/or mass are critical in the development of T2D (Florez, 2008; Petrie et al., 2011). Although Butler et al. (2003) reported that during T2D there is a ≤ 60% reduction in β-cell mass, which is directly correlated with decreased glucose-stimulated insulin secretion (Del Guerra et al., 2005); it has been reported that β-cell function is more critical than β-cell mass in the development of T2D (Ashcroft and Rorsman, 2012). This is

due to the fact that β -cells show resilience and can compensate for insulin demand even when cell numbers are significantly reduced (Cerf, 2013). Thus, several studies have been conducted to understand the β -cell dysfunction and demise. Factors that have been implicated in β -cell dysfunction and demise include: obesity and insulin resistance, cytokine induced inflammation, and overconsumption of saturated fat and free fatty acids (Cerf, 2013).

1.1.5 Prevalence of type 2 diabetes

Presently, people with diabetes in the world are estimated to be more than 382 million (IDF, 2013). Majority of diabetic patients (90-95%) have T2D and this form of diabetes has drastically increased in the last decades (Chen et al., 2011; Gauriguata et al., 2014). In South Africa, more than 9.0% of people aged 30 and above have T2D, representing more than 2 million diabetes cases (Bertram et al., 2013); and although this form of diabetes was previously considered as a disease of the adults, occurrence in children and adolescents has been consistently documented in recent studies over the past decade (Rosenbloom et al., 2008; Pettitt et al., 2014). Moreover, T2D was previously known to be prevalent in developed countries; but a recent study has shown that burden of this disease has drastically increased in developing countries as compared to developed countries (Chen et al., 2011). The drastic increases have been associated with rapid urbanization, which is highly associated with rapid nutrition transition and other lifestyle risk factors for non-communicable diseases (Bertram et al., 2013). Nutrition transition can be defined as a shift in dietary pattern and energy expenditure. In developing countries, rapid urbanization has been associated with transition from traditional high-fiber, low-calorie and low-fat diet toward consumption of westernized high-sugar, high-fat and refined carbohydrate diets (Misra et al., 2013).

1.1.6 Management of type 2 diabetes

T2D is usually managed by 3D formula such as diet, discipline and drug. It is very important to follow a healthy diet along with routine physical exercise to manage T2D. If the disease is not manageable by following proper diets and physical exercise then oral hypoglycemic drugs are usually used to manage the disease. T2D is not well managed in South Africa, with less than 50% of type 2-

diabetic patients achieving glycemic targets and more than two thirds having glycated haemoglobin (HbA1c) concentrations higher than the generally recommended level of 7% (Amod et al., 2012; SEMDSA, 2012). Management of T2D is important to obtain effective control of blood glucose, serum lipid profile and blood pressure, and ultimately reduce the risk of developing long-term complications associated with this disease (ADA, 2013). A proper diet habit or medical nutrition therapy, physical activity and drug or medical therapy are the most common approaches for the management of T2D which are discussed below.

Dietary management and type 2 diabetes

Dietary management is an important factor not only for the effective control of diabetes related parameters, such as blood sugar, blood pressure and blood lipids but also for the management of T2D. Medical nutrition therapy can be defined as a process of treating medical conditions through the use of specifically designed diet. Medical nutrition therapy is highly popular as a therapeutic approach to prevent or delay macrovascular and microvascular complications, as well as to prevent or reduce morbidity and mortality caused by T2D (SEMDSA, 2012).

The medical nutrition plan recommended by the 2012 SEMDSA Guidelines for the Management of T2D focuses mainly on macronutrition and suggests that patients should reduce calorie intake, and consume less saturated fats, trans fat, cholesterol and sodium. The guidelines also recommend that type 2 diabetic patients should limit their intake of sugar to 10% of total energy intake per day (SEMDSA, 2012). Intake of high-calorie sugars, such as sucrose, should be limited because previous studies have consistently linked their consumption to a number of detrimental effects. Sugar consumption, especially in the form of liquid has been associated with increased weight gain (Schulze et al., 2004; Hu and Malik, 2010), visceral adiposity (Dhingra et al., 2007; Hu and Malik, 2010), and risk of developing T2D, cardiovascular disease and metabolic syndrome (Schulze et al., 2004; Dhingra et al., 2007; Hu and Malik, 2010). Thus, nowadays diabetic patients are increasingly substituting sugar with alternative sweeteners in their diets.

Physical activity and type 2 diabetes

Regular physical activity is as important as diet in the management of T2D as it assists in weight loss and maintenance, as well as in controlling blood glucose, lipids and blood pressure (Colberg et al., 2010). Previous studies have consistently shown that both acute and chronic physical exercise have beneficial effects for individuals with T2D. Aerobic exercise is the traditionally prescribed form of physical exercise in T2D management, and has been previously shown to improve insulin sensitivity in patients, as early as the first week of exercise (Winnick et al., 2008). Studies have also shown that physical exercise lowers total and LDL-cholesterol, and increase HDL-cholesterol concentrations (Kadoglou et al., 2007); improves blood pressure (Cohen et al., 2008); reduces the risk of cardiovascular disease (Kokkinos et al., 2009); and plays a profound role in weight loss and managing T2D (Clark et al., 2002). Thus, increased physical activity is highly recommended in the management of T2D in order to prevent or delay the onset of diabetes related complications. According to the SEMDSA guidelines, diabetic patients should perform at least 150 minutes moderately intense aerobic physical exercise per week (SEMDSA, 2012).

Medical therapy for type 2 diabetes

If dietary or medicinal nutrition therapy and physical exercise are not enough for the management of T2D then it is very important to go for the drug therapy. In other words, diet and exercise serve as a first line of therapy and when hyperglycemia persists, anti-diabetic drugs are used. Since diabetes is generally characterized by chronic hyperglycemia; the medications used in diabetes treatment lower glucose concentrations in the blood. The most popular anti-diabetic drugs for T2D are oral hypoglycemic agents, and these can be classified into insulin secretagogues: which improve the amount of insulin secreted by the β -cells of the pancreas; insulin sensitizers: which improve the sensitivity of target tissues to insulin; and alpha-glucosidase inhibitors: which decrease the absorption rate of glucose in the gastrointestinal tract. There are many other oral hypoglycemic drugs available in the market but the above-mentioned drugs are widely used for the management of T2D.

Insulin secretagogues

As stated above these types of anti-diabetic drugs increase the amount of insulin secreted by the β -cells. The most popular class of insulin secretagogues are sulfonylurea compounds. According to Thulè and Umpierrez (2014), these compounds were the first antidiabetic drugs to be used by type 2 diabetic patients. Their mechanism of action involve stimulating the secretion of insulin by the β -cells, as well as having extrapancreatic effects, such as reducing hepatic insulin clearance and decreasing release of glucagon (Thulè and Umpierrez, 2014). Although sulfonylureas are useful in lowering blood glucose in individuals with T2D, these compounds are associated with greater risks of hypoglycemia, as well as weight gain (Rendell, 2004). The other groups of insulin secretagogues can be classified as nonsulfonylureas; and these include meglitinides which also acts by stimulating insulin release from the pancreas but at a binding site different to that of sulfonylureas (Rendell, 2004).

Insulin sensitizers

These oral hypoglycemic drugs lower blood glucose by increasing insulin sensitivity or by decreasing insulin resistance. The most common drugs in this group are biguanides and thiazolidinediones.

The mechanism of action of biguanides include preventing the liver from metabolizing amino acids into glucose (Smith, 2014), and activating adenosine monophosphate kinase, an enzyme that improve the sensitivity of cells to the insulin hormone (SEMDSA, 2012). However, the presence of endogenous insulin is necessary for these mechanisms of action to work (Miller et al., 2013). Under this group of sensitizers, metformin is the most commonly used as it has been found to have less unfavourable side effects compared to other similar drugs (Pernicova and Korbonits, 2014). Biguanides such as phenformin and buformin were used in the past, but were subsequently withdrawn due to undesirable side effects such as the risk of lactic acidosis (Verdonck et al., 1981; Fimognari et al., 2006).

Thiazolidinediones reduce insulin resistance in the insulin sensitive tissues (adipose, muscle and liver) by increasing glucose utilization and decreasing glucose production (Hauner, 2002). Although, the

mechanism by which these compounds exert their effects remains to be fully elucidated; studies have shown that they bind peroxisome proliferate-activated receptor gamma (PPAR γ), a regulatory protein involved in the transcription of glucose and lipid metabolism (Lehmann et al., 1995). Additionally, thiazolidinediones may also improve glucose homeostasis through preservation of β -cell function (Buchanan, 2003). The use of these compounds in the treatment of T2D has been significantly reduced due to the number of adverse health effects. These compounds have been associated with negative cardiovascular outcomes, as well as decreased bone density and increased risks of skeletal fractures (SEMDSA, 2012; Smith, 2014).

Alpha-glucosidase inhibitors

Alpha-glucosidase inhibitors are saccharides that act as competitive inhibitors of alpha-glucosidases, enzymes that are found in the brush border of the small intestine and catalyse the digestion of starch and disaccharides into glucose (Kalra et al., 2014). Thus, alpha-glucosidase inhibitors act by delaying the digestion of carbohydrates in the small intestine, thus reducing and slowing the absorption of glucose (SEMDSA, 2012). These compounds can be classified as anti-diabetic drugs, but they are not necessarily hypoglycemic agents, as they do not act directly on insulin action or sensitivity. Examples of these compounds include acarbose, miglitol and voglibose. According to Kalra et al. (2014), acarbose inhibits both alpha-amylase, as well as other alpha-glucosidase enzymes; whilst miglitol and voglibose inhibit disaccharide-digesting enzymes and do not affect alpha-amylase (Chen et al., 2006). These compounds are used in the management of T2D because they delay carbohydrate absorption in the intestine, and ultimately reduce postprandial blood glucose concentrations (Kalra et al., 2014). In addition, intake of alpha-glucosidase inhibitors has been associated with increased serum insulin, glucagon-like peptide and incretin hormones, as well as reduced body weight (Hoffmann and Spengler, 1997).

Although diet, physical exercise and medical therapy are all important in the management of T2D; excess intake of sugar can cause significant detrimental effects even when the above mentioned factors are well controlled (Johnson et al., 2007). Thus, a number of NNSs are used by diabetic

individuals not only to reduce their total calorie intake but also maintain their blood glucose concentrations at physiologically safer level.

1.1.7 Commonly Used Sweeteners

Although a number of different sweeteners exist, commonly used sweeteners can be classified into natural and artificial sweeteners. Natural sweeteners can further be divided into high and low calorie sweeteners. The details of all types of sweeteners are discussed below.

Natural sweeteners

High calorie natural sweeteners

High calorie sweeteners are a class of natural sweeteners that are associated with high calories per gram; and these include sucrose, fructose and high fructose corn syrup, which provide approximately 4 kcal/g. As indicated earlier, high consumption of these sweeteners has been consistently associated with a great number of metabolic disorders; such as weight gain, dyslipidemia, insulin resistance and finally T2D.

Previous studies have consistently linked the consumption of high calorie containing sweeteners, especially in the liquid form, with increased weight gain and obesity in children (Ludwig et al., 2001), adolescents and adults (Schulze et al., 2004; Hu and Malik, 2010). Although, the mechanism by which dietary sugar causes weight gain has not been fully established; animal studies have shown that consumption of high calorie sweeteners suppressed the secretion of insulin and production of leptin (Stanhope and Havel, 2010). Thus it has been proposed that consumption of sugar in liquid form in humans may not adequately stimulate secretion of insulin and production of leptin, and thus promoting weight gain (Teff et al., 2004).

Moreover, high calorie sweeteners have also been previously associated with visceral adiposity (Dhingra et al., 2007; Hu and Malik, 2010), and risk of developing T2D, cardiovascular disease and metabolic syndrome (Schulze et al., 2004; Dhingra et al., 2007; Hu and Malik, 2010). Other previous clinical and animal studies have indicated that these metabolic disorders occur because, consumption

of high calorie sweeteners increases serum triglycerides (Raben et al., 2002), blood pressure, glucose intolerance and insulin resistance (Johnson et al., 2007).

It has been reported that the active ingredient in causing the above mention disorders is fructose rather than glucose (Nakagawa et al., 2006). It has been reported that fructose may not effectively stimulate insulin and leptin release, and inhibit ghrelin (Stanhope and Havel, 2010); parameters that affect satiety in the central nervous system (Johnson et al., 2007). These changes can significantly increase energy intake, visceral adiposity, and ultimately increase the risk for developing T2D and other metabolic disorders (Dhingra et al., 2007).

Therefore, since high calorie natural sweeteners have been consistently reported to lead cardio-metabolic disorders in animal and clinical studies. Low or calorie natural sweeteners are gaining more popularity.

Low or no calorie natural sweeteners

Low or no calorie natural sweeteners provide sweetness with the benefit of the lower amount calorie per gram; and include sugar alcohols and stevia. A number of sugar alcohols exist and their caloric content ranges from 0.2-2.4 kcal/g; whilst stevia is known to be virtually non-caloric.

Sugar alcohols

Sugar alcohols are a class of polyols, and can be defined as saccharide derivatives that contain multiple hydroxyl groups (Ghosh and Sudha, 2012). These substances are differentiated based on the number of saccharide units present in each molecule (Figure 1.3). Although sugar alcohols are naturally found in minute quantities in some fruits and vegetables, they can be commercially synthesized through hydrogenation of some sugars (Wolever et al., 2002). Figure 1.3 shows the structures of some of the commonly used sugar alcohols: erythritol, isomalt, maltitol, sorbitol, mannitol, and xylitol (Lipinski, 2013). In general, most sugar alcohols are not as sweet as sucrose and have lower caloric content. Moreover, they are partially absorbed in the small intestine and thus have lesser influence on blood glucose compared to sucrose. Additionally, unlike sucrose, sugar alcohols are not cariogenic due to the fact that they are rarely metabolized by microorganisms and far less

insulin is required for their metabolism (Hayes, 2001). Most sugar alcohols have lower sweetness than sucrose and thus larger amounts are required to obtain sweetness levels of sugar. Moreover, majority of these sugar alcohols are associated with laxative effects when used at high concentrations, and according to Ghosh and Sudha (2012), in order to prevent gastrointestinal discomfort, the allowed daily intake for adult is 40-50 g per day and 30 g per day for children.

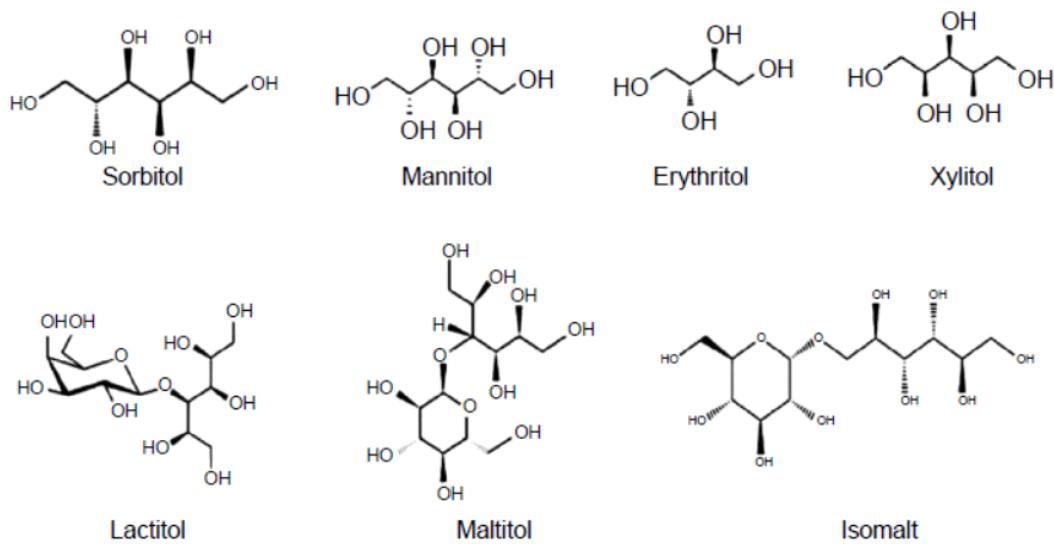


Figure 1.3: Chemical structures of commonly used sugar alcohols (www.intechopen.com)

Erythritol

Erythritol is a four-carbon polyol that was discovered in the year 1848 by John Stenhouse. This sugar alcohol occurs naturally in fruits such as watermelon, pear and grape, and some fermented foods (Shindou et al., 1989). For commercial use as a sweetener, it is produced via fermentation of glucose using either *Trichosporonoides megachiliensis* or *Moniliellapollinis* (Scientific Committee on Food, 2003). The sweetness of erythritol is approximately 60-80% that of sucrose (Scientific Committee on Food, 2003), and has a very low caloric value of 0-0.2 kcal/g (Livesey, 1992). Moreover, more than 90% of erythritol is not metabolized by humans and is excreted unchanged in the urine (Boesten et al., 2013) making it virtually very low-caloric.

Previous studies have suggested that orally-administered erythritol does not influence acute serum glucose and insulin concentrations (Noda et al., 1994). Other studies have shown that this sugar alcohol has hydroxyl radical scavenging activity (den Hartog et al., 2010), reduces lipid peroxidation (Yokozawa et al., 2002) and has endothelial protective effects in diabetic rats (den Hartog et al., 2010). Thus erythritol seems to be suitable for individuals with diabetes. Moreover, erythritol is the only sugar alcohol that has not been associated with laxative side effects (Storey et al., 2007).

Isomalt

Isomalt is chemically composed of a mixture of polyols and hydrolysis of this sugar alcohol yields glucose, mannitol and sorbitol (Dills, 1989). Although this sugar alcohol is derived from sucrose, it has low caloric value which is approximately 2 kcal/g (Livesey et al., 2000). Though not many studies have been conducted to assess the suitability of isomalt for diabetic patients; Gostner et al. (2005) investigated the effects of isomalt consumption in healthy volunteers in a randomized, double-blind, cross-over clinical trial, which had two one month test periods. Their study revealed that consumption of 30 g/kg BW/ day isomalt in diet did not influence serum lipids, fructosamine and leptin (Gostner et al., 2005). According to Gostner et al. (2005), isomalt can serve as a potential sugar substitutes for diabetic patients as its consumption results in reduced postprandial glucose concentrations compared to sucrose. Although issues concerning the laxative effects of isomalt at high concentrations have been reported (Burkitt et al., 1972), further studies suggested that laxative side effects of isomalt only occur at very high concentrations which cannot be matched with typical consumption behaviour (Marteau and Flourie, 2001).

Maltitol

Maltitol is a sugar alcohol derived from maltose and has about 75-90% sweetness of sucrose (Pratt et al., 2011). Commercially, maltitol is usually obtained as maltitol syrup, which is composed of about 50-80% maltitol by weight. Food energy value of this particular sugar alcohol is only 2.1 kcal/g, which is approximately half of that of the high calorie natural sweeteners. Previous studies have shown that substituting sucrose with maltitol lowers glycemic response, glycemic index and insulin concentrations (Quilez et al., 2007) and therefore may be suitable for diabetic patients. Further studies

have reported that the consumption of maltitol reduces fecal pH and increases fecal volume in animal models of male rats (Quilez et al., 2007). Although maltitol is generally safe as sweetener, as with most sugar alcohols, laxative side effects during high consumption remain highly undesirable.

Sorbitol

Sorbitol is a sugar alcohol that is metabolized slowly in the human body and only has 50% the sweetness of sucrose with an energy value of about 2.6 kcal/g. This sugar alcohol is commercially used in a number of sugar-free products, such as baked cakes and cookies (Ghosh and Sudha, 2012). The benefits of sorbitol consumption include: increasing absorption of vitamin B₁₂ and iron in the intestine (Chow et al., 1958; Loria et al., 1962). Other previous studies have also reported that large amounts of sorbitol intake can lead to abdominal discomfort, and mild to severe diarrhea (Islam and Sakaguchi, 2006). A study by Oku and Okazaki (1996) revealed that the laxative threshold of sorbitol for male subjects is 0.66 g/kg body weight, whilst females have a threshold of 0.24 g/kg body weight.

Mannitol

Mannitol is a sugar alcohol that occurs naturally in many fruits and vegetables; e.g. mushrooms, onions, pumpkins etc. (Saha and Racine, 2011) and it is also commercially produced through various chemical and biological methods (Wisselink et al., 2002). Its energy value is 1.6 kcal/g, has approximately 50% sweetness intensity when compared to sucrose, and is widely applied in the medical, pharmaceutical and food industries (Saha and Racine, 2011). According to Shawkat et al. (2012), medical applications of mannitol include management of conditions such as rhabdomolysis as well as raised intracranial pressure. These medical applications are based on studies that have indicated that mannitol has osmotic diuretic properties (Yallop et al., 2008). Mannitol is also a common sweetener and texturing agent in the food and pharmaceutical industries. This sugar alcohol is commonly used in chewing gum as well as chewable tablets due to its low caloric value and ability to mask the unpleasant taste of drugs (Saha and Racine, 2011).

Xylitol

Xylitol is a naturally-occurring sugar alcohol that is found in many fruits and vegetables. This particular polyol is as sweet as sucrose but with lower energy value of 2.5 kcal/g (Rahman and Islam, 2014). Previous studies have suggested that xylitol has a number of beneficial effects, particularly for diabetic patients. Recent studies have shown that consumption of xylitol can ameliorate most of the diabetes related parameters in both normal and T2D rat models (Islam, 2011; Islam and Indrajit 2012), suggesting that this sugar alcohol can be effective in the management of T2D. According to a very recent study by Rahman and Islam (2014), xylitol has also the ability to improve the morphology of pancreatic islets, serum insulin concentration and glucose tolerance level in type 2 diabetic rats. Moreover, other studies have reported that this sugar alcohol is highly effective in the reduction of visceral fat, and therefore improves insulin sensitivity and reduce chances of developing insulin resistance (Amo et al., 2011). In addition, xylitol was also found to have the ability to prevent insulin resistance, induced by non-esterified fatty acid (NEFA) in rats. These studies suggest that xylitol may be effective in preventing or delaying the development of T2D.

Stevia

Stevia is a naturally occurring NNS that is extracted from the leaves of a plant species called *Stevia rebaudiana*. According to Misra et al. (2011), this plant species has been extensively used by Gaurani Indians for more than 150 decades. However, extensive research only began in 1931 when two chemists from France isolated glycosides which give the plant its sweet taste. These compounds can be up to 300 times sweeter than sucrose and the major steviol glycosides include: stevioside, rebaudioside A, rebaudioside C and dulcoside A. Glycosides were first commercialized as sweeteners in 1971, by a Japanese company called Morita Kagaku. Although the whole-leaf and extract of stevia are not approved as generally recognized as safe (GRAS) food additives, steviol glycosides have been approved for use by FDA as food additives, at an ADI of 4 mg/ kg BW/ day.

Stevia and weight gain

Majority of studies have assessed the potential health benefits of stevia in diabetes management. Clinical studies using healthy subjects have indicated that stevia does not influence appetite and food

intake (Anton et al., 2010). Moreover in a study by Melis (1999), administration of 66.7% aqueous stevia extract to normal rats for two months had no influence on body weight gain. Other animal studies in diabetic rats have also reported similar results. In a recent study, 4% stevia whole leaf extract was administered to STZ-induced diabetic rats for five weeks, and revealed no significant differences regarding food intake and body weight gain (Shivanna et al., 2013). On the contrary, according to Figlewicz et al. (2009) 12.5% stevia in drinking water at night, 3 nights/ week for 10 weeks decreases calorie intake, but has no effect on body weight gain in rats. Since the rats were given stevia only at night (3 nights/ week), the authors hypothesized that the treated rats may have been overeating on days when they were not given stevia, and therefore compensated for overall food intake. Therefore, majority of studies indicate that stevia consumption is not associated with changes in body weight.

Effect of stevia on glucose homeostasis

Stevia has been consistently shown to have hypoglycemic effects in both clinical and animal studies. An acute clinical study by Anton et al. (2010^b) assessed the effects of preloads containing stevia before meal on postprandial serum glucose and insulin concentrations. The results of their study indicated that eating crackers sweetened with stevia prior to the main meal, lowers both postprandial glucose and insulin concentrations when compared to sucrose (Anton et al., 2010). Animal studies have previously indicated that consumption of stevia extracts and stevia whole leaf powder at both low and high concentrations reduces serum glucose concentrations and improves glucose tolerance in both diabetic and normal rats (Sharma et al., 2012; Mohd-Radzaman et al., 2013; Shivanna et al., 2013). It has been suggested that stevia reduces blood glucose by its strong antioxidant properties (Mohd-Radzaman et al., 2013). Moreover, an in vitro study recently assessed the effect of four different stevia extracts on glucose transport activity in HL-60 human leukaemia and SH-SY5Y human neuroblastoma cells. The results indicated that stevia is able to enhance glucose uptake in both cell lines as efficiently as insulin (Rizzo et al., 2013). On the contrary, in a chronic animal study by Figlewicz et al. (2009), rats were administered with beverages sweetened with 12.5% stevia and results showed no significant difference in terms of fasting insulin concentrations and intravenous

glucose tolerance test, when compared to the water consuming control group (Figlewicz et al., 2009). The differences in the results of the above-mentioned studies might be due to different experimental conditions. However, it has no doubt that stevia has strong hypoglycemic as well as anti-diabetic effects. Although a number of previous studies reported the effects of various extracts and compounds from stevia on normal and diabetic rats, the effects of commercially available stevia in these regards are still unknown.

Stevia and diabetes-related complications

Since stevia is anti-hyperglycemic and has antioxidant properties, it is expected that this natural NNS has significant potential in preventing diabetes-related complications. The effects of stevia administration on kidney function in normal animals have been reported. In a very recent study, Hashemi et al. (2013) assessed the potential effects of stevia administration on oxidative stress in kidney of BALB/c mice. The mice were acutely treated with 700 mg/kg BW Rebadioside A (Reb A), the main component of stevia extract. The results suggested that a single dose of Reb A has protective effects against oxidative stress in the kidney (Hashemi et al., 2013). On the contrary, Melis (1995) reported that chronic intake of stevia at 66.7% may have detrimental effects. The study evaluated the effects of 66.7% stevia extract (66.7 g dried leaves/ 100 ml water) in normal rats for eight weeks, on renal function and blood pressure. Treatment with stevia induced systemic and renal vasodilation leading to hypotension (Malis, 1995).

A recent study investigated the effect of stevia aqueous extract on oxidative stress in alloxan-induced type 2 diabetic rats. The rats were administered with 250 mg/ kg BW for 28 days, and the results of hepatic antioxidant enzymes activity and lipid peroxidation levels clearly demonstrated that stevia extract has significant antioxidant potential in diabetic rats (Sharma et al., 2012). In a more recent study, Shivanna et al. (2013) evaluated the influence of stevia in the treatment of diabetes. Administration of stevia to the diabetic rats improved liver and kidney histology (Shivanna et al., 2013). The results of the most of the above-mentioned studies have clearly shown that consuming stevia is beneficial in the management of diabetes.

1.1.8 Artificial sweeteners

Although a number of alternative sweeteners exist, artificial sweeteners have become the most popular amongst diabetic individuals (Sylvetsky et al., 2012). The sweetness of artificial sweeteners is known to range from 30 to 13000 times in comparison to sucrose (Shanker et al., 2013). Thus, due to their intense sweetening power, very small proportions are required to obtain the sweetness of sucrose, allowing their products to be labelled as non-nutritive (Zyglar et al., 2011). Artificial sweeteners are extensively used in numerous drinks and food products including diet yogurts, soft drinks and chewing gum (Gardner et al., 2012). Moreover, artificial sweeteners are also widely used as commercially available sugar substitutes, where they are often used as a blend of different sweeteners in order to obtain a sweetness profile similar to the table sugar, sucrose (Gardner et al., 2012). Since other sweeteners are often associated with adverse health outcomes when used in excess and require large amounts to obtain the sweetness level of sucrose, both food manufacturers as well as consumers are increasingly becoming interested in the use of artificial sweeteners as alternative sugar substitutes (Sylvetsky et al., 2012). Five artificial sweeteners have been approved by the United States Food and Drug Administration (USFDA) for use as food additives and these include: aspartame, sucralose, saccharin, acesulfame potassium (Ace-K) and neotame (Neacsu and Madar, 2014). Although, several studies have been conducted to assess whether artificial sweeteners are safe for consumption by humans; majority of the studies have reported conflicting results. Nonetheless, artificial sweeteners are strongly recommended for both obese and diabetic individuals in order to avoid excessive intake of calories. Thus, a number of diabetic patients are increasingly substituting calorie-containing sweeteners with artificial sweeteners (Gougeon et al., 2004). The principle behind the use of artificial sweeteners is that the patients can enjoy a wide variety of sweetened products without the adverse impact on blood glucose control, which is associated with sugar. The effects of each commonly used artificial sweetener on different diabetes related parameters are discussed in detail below.

Saccharin

Saccharin is the oldest artificial sweetener and was first synthesized by Constantin Fahlberg in 1878, at Johns Hopkins University (Kauffman and Priebe, 1978). The United States Department of Agriculture (USDA) began investigating saccharin as early as 1907, and presently this sweetener is used in more than 100 countries, including South Africa, with an ADI of 5 mg/ kg BW/ day as set by the USFDA. Saccharin is approximately 300 times sweeter than sucrose, and is used to sweeten many food products, drinks, toothpastes, medicines, and as a table top sweetener (Touyz, 2011). According to Singh (2013), this artificial sweetener is highly popular due to stability at high temperatures, long shelf life and low cost.

Saccharin and weight gain

Although saccharin is one of the most studied artificial sweeteners in relation to its effects on body weight, reports have been largely conflicting. A number of previous animal studies have suggested that administration of saccharin increases body weight. In some previous studies, yogurt sweetened with 0.3% saccharin was associated with higher body weight gain compared to yogurt sweetened with either 20% sucrose or glucose in male Sprague-Dawley rats (Swithers and Davidson, 2008; Swithers et al., 2009; Swithers et al., 2013). In these studies, increased weight gain was linked to increase in food and caloric intake, and diminished caloric compensation (Swithers and Davidson, 2008). However, a study by Feijo et al. (2013) also revealed that yogurt sweetened with 0.3% saccharin caused more weight gain in rats, compared to 20% sucrose. In their study, caloric intake was similar among the groups and it was suggested that more weight gain in saccharin consuming group might be due to lower energy expenditure. Moreover, in a study by Andrejic et al. (2013), six weeks feeding of 0.0005% saccharin solution ad libitum, in male and female rats, increased body weight, but decreased food intake compared to normal drinking water. In contrary, supplementation of 0.1% saccharin in drinking water for two hours a day for three weeks did not show any effect on food intake and weight gain, in both male and female Sprague-Dawley rats (Park et al., 2010). Thus the effects of saccharin on food intake, body weight gain and glucose homeostasis remains unclear.

Effect of saccharin on glucose homeostasis

Although studies have extensively assessed both acute and chronic effects of saccharin on blood glucose, insulin and other parameters related to glucose homeostasis; it is still unclear whether saccharin intake is effective in glycemic control. Majority of clinical studies have suggested that saccharin is biologically inert and does not affect glucose homeostasis (Bryant et al., 2014). Nonetheless, Just et al. (2008) examined whether taste stimulation by saccharin has any effect on cephalic phase insulin release, and revealed that saccharin intake increases plasma insulin concentration in healthy humans (Just et al., 2008).

Interestingly, in a series of studies conducted by Swithers and others, it was revealed that 0.3% saccharin solutions/sweetened yogurt impairs more glucose homeostasis when compared to 20% glucose or sucrose solutions/sweetened yogurt (Swithers and Davidson, 2008; Swithers et al., 2009; Swithers et al., 2010; Swithers et al., 2013). This has been recently shown to be dependent on taste stimulation and to occur through impairing glucagon-like peptide-1 (GLP-1) release (Swithers et al., 2012; Swithers et al., 2013). In contrary, some studies have also reported that saccharin intake causes a decrease in blood glucose concentrations in normal female albino rats (Abdelaziz and Ashour, 2011). Thus, the authentic effects of saccharin intake on glucose homeostasis and diabetes related complications are remained highly debatable.

Saccharin and diabetes-related complications

The acute and long-term toxicological effects of saccharin have also been conducted in several animal and clinical studies. In a previous study, administration of 35 mg/kg BW/ day of saccharin for 35 days decreased serum triglycerides, cholesterol, albumin and total protein concentrations in female rats. Moreover, saccharin intake was also associated with increased inflammatory biomarkers, namely creatinine, alkaline phosphatase, alanine transaminase and aspartate transaminase in the serum (Abdelaziz and Ashour, 2011). In accordance with the above mentioned study, several studies have suggested that oral administration of saccharin can elevate serum ALT, AST and ALP in normal rats and normal humans (Negro et al. 1994; Abdelaziz and Ashour, 2011; Andrejic et al. 2013). These abnormalities in serum enzymes might be due to abnormally high doses (e.g. 35 mg/kg BW/ day) of

saccharin used in these studies, which led to changes in liver histology and ultimately leakage of these enzymes to the blood (Andrejic et al. 2013). Thus, high intake of saccharin seems to cause tissue damage in animals and this ultimately increases inflammatory biomarkers in the serum.

Aspartame

Aspartame is a dipeptide artificial sweetener that is trademarked by different companies like NutraTaste, NutraSweet and Equal (Ashok et al., 2014). This particular artificial sweetener was first synthesized in 1965 and was approved by the USFDA in 1981 at an ADI of 50 mg/ kg BW/ day (Rencuzogullari et al., 2004). Unlike other artificial sweeteners aspartame is metabolized to produce methanol, aspartic acid and phenylalanine; common dietary metabolites (Butchko et al., 2002). Although aspartame has the same calories as sucrose on weight to weight basis, it is considered virtually calorie-free because it is approximately 200 times sweeter than sucrose. Thus, almost 200 times less amount is needed to obtain the same sweetness level (Magnuson et al., 2007). Currently, aspartame is one of the most popular artificial sweeteners in the world and is used in more than 6000 different food products and drinks, and in many pharmaceutical products (Rencuzogullari et al., 2004). The detailed effects of aspartame on body weight gain, glucose homeostasis and diabetes-associated complications are discussed below.

Aspartame and weight gain

Similar to saccharin, aspartame has been studied extensively but its effects on food intake and body weight gain remain unclear. A study by Hunty et al. (2006) reviewed clinical evidences for the influence of aspartame on energy intake, weight loss and weight maintenance in normal humans. In their review, it was concluded that aspartame administration is strongly associated with reduced energy intake and decreased weight gain when compared to sucrose control, but not compared to water (Hunty et al., 2006). Nonetheless, majority of the previous animal studies have reported contrary results. Collison et al. (2012) recently conducted a long term study which aimed at investigating the effects of lifetime exposure of aspartame beginning in utero. In their study, normal mice were administered with a 0.25 g/L aspartame in drinking water ad libitum and the results suggested that aspartame exposure increased food intake and body weight gain at 17 weeks of age

(Collison et al., 2012). In a more recent study by Feijo et al. (2013), administration of yogurt sweetened with 0.3% aspartame in normal rats for 12 weeks caused a significant elevation of body weight compared to the control rats receiving yogurt sweetened with 20% sucrose. Interestingly, in their study caloric intake was similar in both groups (Feijo et al., 2013). Thus, the effects of aspartame on food intake and body weight gain are still conflicting.

Effect of aspartame on glucose homeostasis

Clinical studies have indicated that aspartame intake does not alter serum glucose and insulin concentrations (Carlson and Shah, 1989; Anton et al., 2010), when some previous animal studies have reported different results. In a recent study, Collison et al. (2012^a) investigated the effects of chronic lifetime consumption of aspartame in C57BL/6J mice, beginning at utero. The study reported that the consumption of 50 mg/kg BW aspartame per day increased fasting blood glucose concentrations and decreased insulin sensitivity during an insulin tolerance test, at 17 weeks of age (Collison et al., 2012^a). Further experiment also suggested that the presence of monosodium glutamate worsen the glucose-insulin homeostasis of aspartame in C57BL/6J mice (Collison et al., 2012^b).

Aspartame and diabetes-related complications

Although majority of studies have focused on the effects aspartame on neurotoxicity (Bergstrom et al., 2007; Ashok et al., 2014), Abhilash et al. (2011) reported that chronic consumption of aspartame may damage the liver, when administered at 1000 mg/kg BW for 180 days in rats. The liver damage in their study was also confirmed by increased concentrations of liver function enzymes which were corroborated with histopathological findings (Abhilash et al., 2011). Since aspartame is metabolized into phenylalanine, aspartate and methanol (Christian et al., 2004); tissue damage may be attributed to increase of methanol in the serum. Methanol has been previously shown to cause changes in the surface charge density as well as oxidative stress, leading to leakage of cellular enzymes (Parthasarathy et al., 2006). Hence, consumption of aspartame may not be suitable after a certain dose level.

Sucralose

Sucralose was discovered in 1976 during research collaboration between Tate & Lyle and the Queen Elizabeth College in London. Sucralose was first approved for consumption in Canada in 1991 and was approved by the USFDA in 1998 at an ADI of 5 mg/ kg BW/ day (FDA, 1998). By the year 2008, this NNS was approved as a food additive in more than 80 countries including South Africa (Schiffman and Rother, 2013). Sucralose is a NNS that is synthesized by selectively substituting the hydroxyl groups of sucrose with chlorine groups. The resulting product is a molecule that is approximately 600 times sweeter than sucrose (Binns, 2003). Sucralose is advantageous because its high heat-stability allows it to be useful as a sweetener during cooking and baking. Moreover, sucralose remains stable even at low pH allowing it to be used in products with long periods of storage (Binns, 2003). Effects of sucralose on body weight gain, glucose homeostasis and diabetes-related complications are given below.

Sucralose and weight gain

A number of clinical studies have been conducted to assess whether sucralose affects food intake, appetite and ultimately body weight gain or not. Baird et al. (2000) conducted a study aimed to assess the sucralose tolerance in healthy subjects. In their study, healthy human subjects were given sucralose at a dose of up to 500 mg/kg/day, for a period of 13 week. The results of the study indicated that consumption of sucralose has no effect on food intake and weight gain (Baird et al., 2000). Other clinical trials have also reported similar results. In an acute study, where 0.1% sucralose in drinking water was given to female human subjects before a meal, there were no significant differences with regards to appetite and food intake (Brown et al., 2011). Moreover, Ford et al. (2011) conducted an acute randomized, single-blind, crossover study in healthy human subjects. The subjects were administered with 50 ml of 0.083% sucralose before a buffet meal, and the study revealed no influence on appetite ratings and food intake (Ford et al., 2011). Thus, the acute and chronic studies conducted on human subjects suggest that sucralose consumption has no influence on appetite, energy intake and ultimately body weight gain.

Effect of sucralose on glucose homeostasis

Although, a number of clinical studies have consistently suggested that both acute and chronic consumption of sucralose has no effects on insulin, GLP-1, glucagon, and glucose homeostasis; recent animal studies have reported contradictory results (Grotz et al., 2003; Brown et al., 2011; Ford et al., 2011). Saada et al. (2013) recently investigated the effects of sucralose administration in normal and STZ-diabetic rats at a daily dose of 11 mg/kg BW for six weeks. Their results indicated that sucralose administration reduces serum glucose and has no effects on serum insulin concentrations (Saada et al., 2013). On the contrary, other studies using rats have reported that sucralose consumption enhances glucose absorption and ultimately increase blood glucose concentrations, via stimulating apical availability of GLUT-2 (Ma et al., 2010). Hence, the effects of sucralose on glucose homeostasis are still contradictory.

Sucralose and diabetes-related complications

The toxicological and carcinogenic effects of chronic consumption of sucralose have been evaluated in several previous studies (Mann et al., 2000; Rodero et al., 2009). However, the studies directly evaluating the effects of sucralose administration on diabetes-related parameters are limited. In a chronic study, normal rats were given sucralose in the diet ranging from 750-300 mg/kg BW/ day for 26 weeks, the treated rats were subjected to relative increase in kidney weight, and weight and size of the cecum. Interestingly, there were no changes in inflammatory biomarkers and the rats showed no signs of toxicity (Rodero et al., 2009). On the other hand, the effects of sucralose on serum lipid profile are conflicting. The clinical trial by Baird et al. (2000) reported that supplementation of sucralose diet at 500 mg/kg BW/ day for 13 week has no effects on serum lipid profile in normal human subjects, when some animal studies have reported the opposite results. In a recent study by Saada et al. (2013), supplementation of sucralose at a dose of 11 mg/kg BW/ day to diabetic rats decreased triglycerides, and elevated total cholesterol, LDL-cholesterol and HDL-cholesterol. From the results of this study, it has been suggested that the changes in serum triglycerides may be attributed to the deactivation of lipoprotein synthase due to insulin deficiency, whilst increase in cholesterol might be caused by increased intestinal absorption of dietary cholesterol (Saada et al.,

2013). The differences in the results of these previous studies may be due to different experimental designs and doses used.

Cyclamate

Cyclamate was discovered by Michael Sveda from the University of Illinois, in the year 1937. It is synthesized via sulfonation of cyclohexylamine to yield a product that is about 30 to 50 times the sweetness of sucrose. Thus, cyclamate is the least potent NNS and therefore greater amounts are added to achieve similar sweetness levels compared to the other artificial sweeteners.

Although cyclamate was approved as a food additive in the United States in 1958, safety issues began in 1969 when a study reported that the high doses of cyclamate (10:1 ratio with saccharin) increases the incidence of bladder cancer in male rats (Price et al., 1970). This was followed by banning of cyclamate by the United States Food and Drug Administration (USFDA) in 1970, thus this artificial sweetener is still banned in the USA and other parts of the world to this day. Nonetheless, the mechanism by which cyclamate causes cancer in rats was identified to be a rat specific phenomenon (Oser et al., 1976). Thus, since no studies have reported an association between cyclamate and cancer in humans, this NNS has been approved by the European Scientific Committee on Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at an acceptable daily intake of 11 mg/kg, and is used as a food additive in more than 50 countries, including South Africa (Yang, 2010).

Studies evaluating the safety of cyclamate

Cyclamate is not approved by the USFDA and thus studies assessing the safety of this artificial sweetener are greatly limited. After cyclamate was banned in the USA and other parts of the world, majority of studies began to focus on the mechanism by which cyclamate causes cancer in rats. Previous studies have shown that although cyclamate is not toxic, it can be converted by bacterial flora in the gastrointestinal tract into cyclohexylamine, a very toxic metabolite which is responsible for causing cancer in rats (Price et al., 1970). Oser et al. (1976) reported that cyclamate is converted into cyclohexylamine at a much higher rate in rats than in rabbits and humans due to the different types of microorganisms in the gut of each species. According to Oser et al. (1976), cyclamate is

mainly metabolized into cyclohexylamine by clostridia in rats, by enterobacteria in rabbits, and enterococci in humans (Oser et al., 1976).

Subsequent studies in humans have shown that conversion of cyclamate into cyclohexylamine varies. Some people are quick converters, whilst the majority convert cyclamate at a very lower rate (Buss et al., 1992). In a clinical study by Buss et al. (1992), conversion of cyclamate into cyclohexylamine was investigated in healthy human subjects provided with 1 g cyclamate per day for one week, and cyclohexylamine was measured in the urine. The results showed that more than 78% of volunteers did not convert cyclamate to cyclohexamine (Buss et al., 1992).

Although majority of people do not convert cyclamate, there is concern about the small percentage of people who are quick converters. Thus, Renwick et al. (2004) evaluated the effect of long-term administration of cyclamate on its conversion into cyclohexylamine, using high converters as subjects. The subjects were supplied with cyclamate 250 mg/kg BW three times daily for 13 week. The results showed no toxic effects on the volunteers and it was concluded that cyclamate is not toxic in humans when consumed at the recommended daily intake level (Renwick et al., 2004).

Thus, the toxic effect of cyclamate appears to be a rat-specific phenomenon. In support of this statement, a study by Martins et al. (2005) investigated the effects of consuming cyclamate during pregnancy on the rat foetal liver. The pregnant rats were supplied with cyclamate at 60 mg/kg BW for 5 days, and it was revealed that cyclamate is highly toxic in the liver of rat foetuses (Martins et al., 2005). From the information of the above-mentioned studies, the toxicological effect of cyclamate is still conflicting.

1.2 Rationale of the study

Non-calorie or non-nutritive sweeteners (NNSs) (artificial sweeteners and stevia) have gained a lot of popularity in the food industry because they are many times sweeter than sugar and thus only require relatively smaller amounts to achieve the sweetness level of sucrose. Moreover, NNSs are strongly recommended for use as sugar substitutes for individuals with T2D (SEMDSA, 2012) simply

because unlike sugar these type of sweeteners theoretically do not influence blood glucose concentrations when ingested. Nonetheless, previous animal studies have reported conflicting results, and thus the authentic effect of consuming artificial sweeteners in T2D remains highly debatable. Moreover, most previous studies have used NNS in their pure form and not in the form that is consumed by people. Recent animal studies have indicated that NNSs may have synergistic or additive effects when used with other food additives (Collison et al., 2012; Lau et al., 2006). Therefore, since commercially available NNSs are used in combination to achieve the sweetness profile of sucrose, and sometimes contain other biologically active ingredients such as lactose and glucose; we hypothesized that commercially available NNSs may have different effects to what has been previously reported.

1.3 Aims of the study

- I. The present study primarily aimed at investigating the effects of commercially available NNSs in an experimentally-induced rat model of T2D.
- II. The study also assessed toxicological effects of these NNSs at a higher dose in normal rats.

1.4 Objectives

- I. Determination of the effects commercially available NNSs on food and fluid intake, and body weight gain.
- II. Investigating the effects of commercially available NNSs on glucose homeostasis and other diabetes-related parameters.
- III. Assessing the effects of commercially available NNSs on diabetes-related complications organ tissue damage.

CHAPTER TWO

Materials and methods

2.1 Animals, housing and feeding

Seven-week-old male Sprague-Dawley rats were procured and maintained at the Biomedical Resource Unit (BRU) of the University of KwaZulu-Natal at Westville Campus, Durban, South Africa. The rats were randomly divided into eleven groups, as follows: Normal Control (NC), Diabetic Control (DBC), Diabetic Aspartame (DASP), Toxicological Aspartame (TASP), Diabetic Sucralose (DSCL), Toxicological Sucralose (TSCL), Diabetic Cyclamate (DCLM), Toxicological Cyclamate (TCLM), Diabetic Saccharin (DSAC), Toxicological Saccharin (TSAC) and Diabetic Stevia (DSTV) with 5-7 rats in each group (Table 2.1). Animal groups in the diabetic study (DASP, DSCL, DCLM, DSAC, DSTV) were administered with their respective NNS solutions at concentrations equivalent to the sweetness of 10% sucrose solution, whilst the animal groups in the toxicity study (TASP, TSCL, TCLM, TSAC) received their respective NNS solutions at concentrations equivalent to the sweetness of 20% sucrose solution. The toxicological groups were supplied with a two times higher concentration of NNS compared to the diabetic groups because diabetic rats have been reported to drink approximately twice the volume of normal rats (Wilson and Islam, 2012). Thus, different concentrations were used in order to maintain similar level of consumption in treated groups. The rats were housed as maximum of two per medium-sized polycarbonated cage, in a room with 12 hour light-dark cycle, and well controlled temperature and humidity. They were fed commercially available standard rat pellet diet *ad libitum* throughout the 13 week experimental period. Animal procedures in the present study were performed in accordance with the rules and regulations of the University of KwaZulu-Natal Animal Ethics Committee (Ethical approval number: 003/13/Animal).

Table 2.1: Grouping of rats and types of NNS solutions

Group	Name	Number of rats	Drinks (% Sucrose equivalent)
NC	Normal Control	Six	Water
DBC	Diabetic Control	Five	Water
DASP	Diabetic Aspartame	Six	Aspartame (10% equivalent)
TASP	Toxicological Aspartame	Five	Aspartame (20% equivalent)
DSCL	Diabetic Sucralose	Seven	Sucralose (10% equivalent)
TSCL	Toxicological Sucralose	Five	Sucralose (20% equivalent)
DCLM	Diabetic Cyclamate	Six	Cyclamate (10% equivalent)
TCLM	Toxicological Cyclamate	Five	Cyclamate (20% equivalent)
DSAC	Diabetic Saccharin	Six	Saccharin (10% equivalent)
TSAC	Toxicological Saccharin	Five	Saccharin (20% equivalent)
DSTV	Diabetic Stevia	Six	Stevia (10% equivalent)

2.2 Induction and confirmation of diabetes

Insulin resistance and β -cell dysfunction are the major characteristics of Type 2 Diabetes (T2D).

Thus, T2D was induced as described previously by Wilson and Islam (2012). Briefly, the rats were administered with 10% fructose (Nature's choice, Wholefood Specialist, Meyerton, South Africa) solution *ad libitum*, for two weeks to induce insulin resistance. Then they were fasted overnight for 12 hours and injected (i.p.) with a freshly prepared streptozotocin (STZ) (Sigma-Aldrich, St. Louis Missouri, USA) solution (40 mg/kg bw) dissolved in 0.1 M citrate buffer (pH 4.4) to induce partial β -

cell destruction. Rats in the normal control and normal toxicological groups were supplied with normal drinking water and injected with vehicle buffer instead of 10% fructose and STZ solution, respectively. One week after the STZ injection, blood was collected from the tail vein of each animal, and blood glucose was measured using a portable glucometer (GlucoPlus, Quebec, Canada). Rats with three hour fasting blood glucose (3h-FBG) concentrations ≥ 300 mg/dl were considered as diabetic.

2.3 Measurement of food and fluid intake, and body weight gain

Food and fluid intake were measured daily throughout the 13 week experimental period. Both daily food and fluid intake were determined by subtracting the amount left at the end of each day from the given amount, and then divided by the number of rats in each cage to calculate the consumption of each animal per day. Body weight of each animal was measured weekly, and weight gain of each animal at the end of the 13 week experimental period was calculated by subtracting the initial body weight from the final body weight.

2.4 Non-fasting blood glucose and the oral glucose tolerance test

Non-fasting blood glucose was measured weekly over the 13 week experimental period. In this regard, all rats were fasted for 3 hour and blood glucose was measured from the tip of the tail vein using a portable glucometer (Gluco-Plus, Quebec, Canada). During the 3 hour fasting period, the rats were supplied with normal drinking water, and food was immediately returned to the rats after glucose measurement.

The oral glucose tolerance test (OGTT) was performed at the 12th week of the experimental period. A D-glucose solution was prepared by dissolving into distilled water at a concentration of 1 g/ml. In order to perform the test, all rats were fasted overnight for 12 hours and then orally administered a D-glucose solution (2 g/kg bw). Subsequently, blood glucose was measured at 0 (prior to oral glucose administration), 30, 60 and 120 min after the oral glucose dose. During the 12 hour fasting period, the rats were supplied with normal drinking water, and food was supplied immediately after the OGTT.

2.5 Collection of blood and tissue samples

At the end of the experimental period, rats were fasted overnight for 12 hours and then sacrificed using halothane anaesthesia. Whole blood sample of each animal was collected via cardiac puncture into 14 ml Falcon tubes (BD Biosciences, Franklin Lakes, NJ, USA) and immediately stored on ice for 3 hours to coagulate. After this period, the blood was centrifuged at 3000 rpm for 15 minutes and serum was separated immediately and stored at -30 °C for further analysis.

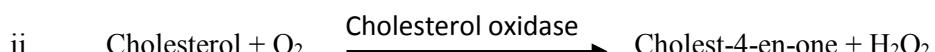
The heart, liver, brain and kidney of each animal was collected, washed in normal saline (0.9% sodium chloride), wiped and weighed when the pancreas was only collected and weighed. A small piece of each organ sample was cut and immediately stored in 10% phosphate buffered formalin at room temperature, for histopathological examination. The formalin was replaced every week during the entire period of preservation.

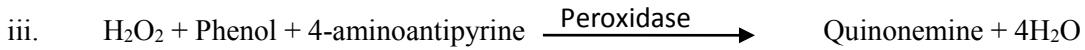
2.6 Serum analysis

The serum stored at -30 °C was slowly thawed by placing the samples on ice. An Automated Chemistry Analyzer (Labmax Plenno, Labtest Co. Ltd., Lagoa-Santa, Brazil) was used to determine the concentrations of serum alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine kinase (CK-MB), creatinine, fructosamine, uric acid and serum lipids. The principles of analyses for the above-listed parameters are briefly described below.

2.6.1 Serum lipid profile

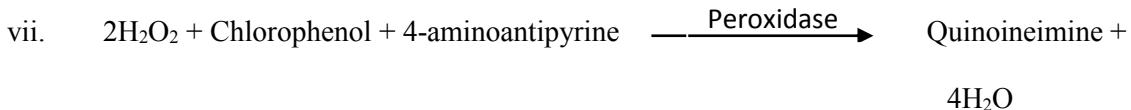
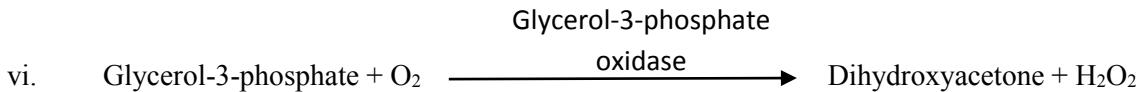
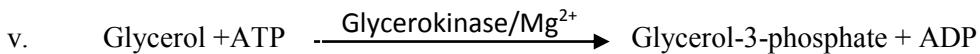
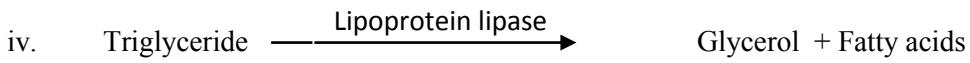
Total cholesterol: Total cholesterol was measured directly in the serum according to the following reactions:





Cholesterol ester was hydrolysed, by an enzyme called cholesterol esterase, into cholesterol and free fatty acids (I). The cholesterol was then oxidized into Cholest-4-en-one and hydrogen peroxidase, by cholesterol oxidase (II). The resulting hydrogen peroxidase reacted with phenol and 4-aminoantipyrine, in a reaction catalysed by peroxidase to produce quinonemine (III). Quinonemine is a red coloured complex that absorbs maximum light at a wavelength of 500 mn. Thus, the intensity of the red colour is directly proportional to the cholesterol concentration in the serum sample.

Triglycerides: The principle for measuring serum triglyceride involved the following reactions:



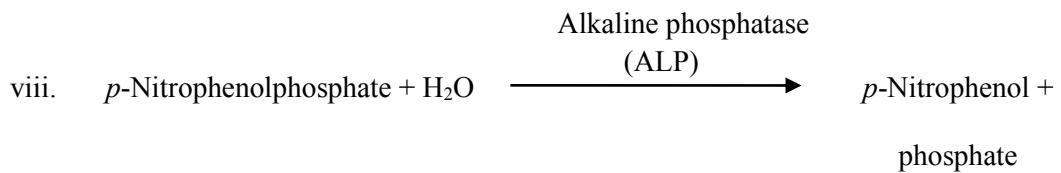
As shown in reaction (IV), serum triglyceride was metabolized by lipoprotein lipase into glycerol and free fatty acids. The resulting glycerol was phosphorylated into glycerol-3-phosphate in the presence of ATP and magnesium ions, by the enzyme glycerokinase (V). Glycerol-3-phosphate was then oxidized into dihydroxyacetone and hydrogen peroxide, catalyzed glycerol-3-phosphate oxidase (VI). Finally, the resulting hydrogen peroxide was reacted with chlorophenol and 4-aminoantipyrine and produced quinonemine and water, and this reaction was catalysed by peroxidase (VII). The resulting quinonemine was then used to determine the concentration of serum triglyceride, as described for total cholesterol above.

HDL-Cholesterol: HDL-cholesterol was measured by selectively precipitating LDL-cholesterol and very low density lipoproteins (VLDLs) in the serum, using phosphotungstic acid and magnesium chloride. The supernatant was then separated via centrifugation at 3500 rpm for 15 minutes. HDL-cholesterol was then measured using the same principle described above for total cholesterol.

LDL-cholesterol was calculated using the following formula: LDL-Cholesterol (mg/dl) = [Total Cholesterol (HDL Cholesterol + triglycerides/5)] when ‘triglycerides/5’ is equivalent to the VLDL-cholesterol.

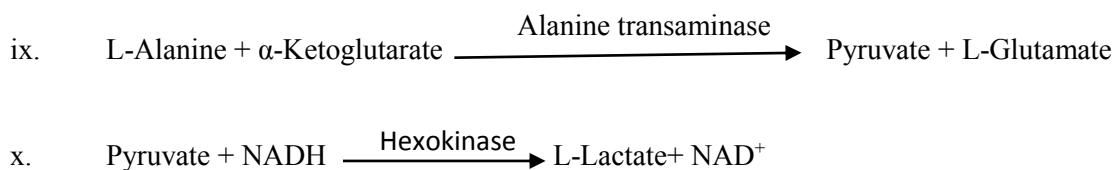
2.6.2 Enzymes in the serum

Alkaline phosphatase (ALP): The following reaction is involved in the determination the concentration of serum alkaline phosphatase (ALP):



ALP hydrolysed *p*-Nitrophenolphosphate under alkaline condition and yielded *p*-Nitrophenol and phosphate, the level of *p*-Nitrophenol produced was proportional to the concentration of the enzyme and the absorbance was read at 405 nm.

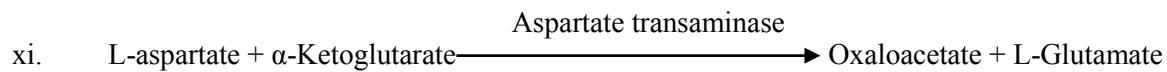
Alanine transaminase (ALT): Serum alanine transaminase was measured according to the following test principle:



As presented in reaction (IX) Alanine transaminase (ALT) transferred the amino group from alanine to α -Ketoglutarate and yielded pyruvate and L-glutamate. The resulting pyruvate was then converted to

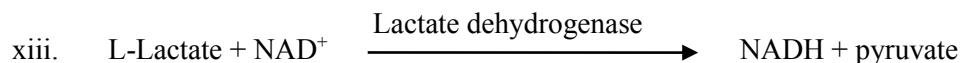
L-lactate in the presence of NADH which was oxidized and yielded NAD⁺ (X). The absorbance of NAD⁺ was thus directly proportional to the concentration of ALT.

Aspartate transaminase (AST): Determination of aspartate transaminase used the following reactions:



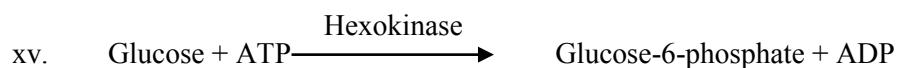
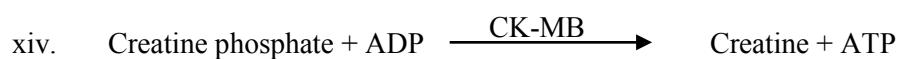
Reaction XI shows that AST transferred the amino group from aspartate to α -Ketoglutarate to form oxaloacetate and L-glutamate. The produced oxaloacetate was then converted by the malate dehydrogenase enzyme to malate in the presence of NADH, resulting to the oxidation of NADH to NAD⁺ (XII). Thus, the absorbance of NAD⁺ was thus directly proportional to the concentration of AST.

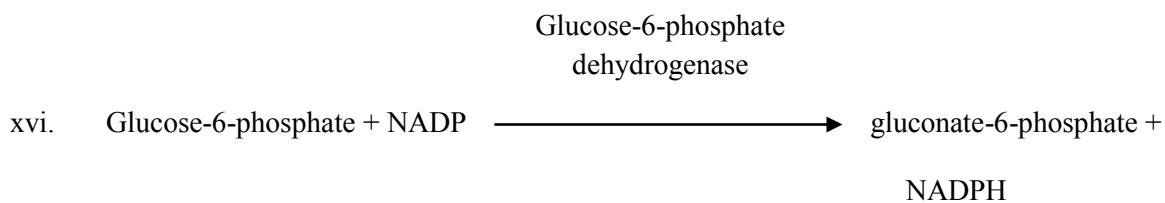
Lactate dehydrogenase (LDH): The following test principle was used to determine the concentration of serum lactate dehydrogenase (LDH):



Pyruvate or lactate can be used as a substrate in this test (XIII). LDH catalyzed the oxidation of lactate to pyruvate in the presence of NAD⁺. The absorbance of the resulting NADH was measured at 340 nm and was directly proportional to LDH concentration.

Creatine kinase (CK-MB): The principle of the procedure for measuring Creatine Kinase (CK-MB) was as follows:



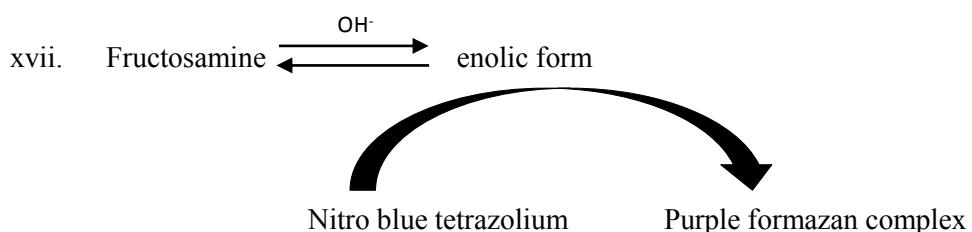


As shown in reaction XIV, creatine kinase catalyzed the conversion of creatine phosphate to creatine. In this first reaction ATP was produced and used in the phosphorylation of glucose which yielded glucose-6-phosphate (XV). Finally, this product was reduced to gluconate-6-phosphate, with the production of NADPH, in a reaction catalyzed by glucose-6-phosphate dehydrogenase. The amount of NADPH produced was detected by spectrophotometric absorption at 340 nm, and was proportional to the concentration of CK-MB in the serum sample.

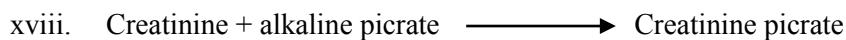
2.6.3 Serum fructosamine, creatinine and uric acid

Serum fructosamine: Serum fructosamine was determined using the following test principle:

At alkaline pH fructosamine converted to an enolic form which reduced nitro blue tetrazolium to a “purple formazan” complex (XVII). The absorbance difference after incubation for 10 minutes and 15 minutes was directly proportional to the level of fructosamine in the serum sample.

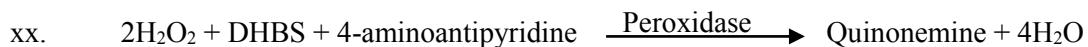


Serum creatinine: The following test principle was used to measure serum creatinine concentration:



The test involved the above reaction which yielded creatinine picrate complex (XVIII). This complex was red in colour and its intensity measured at 510 nm was directly proportional to the concentration of serum creatinine.

Serum uric acid: The following two reactions are involved in determining the concentration of serum uric acid:



The quinonemine yielded a red coloured product and the absorbance was measured between 490-540 nm. The colour intensity was directly proportional to the concentration of serum uric acid.

2.6.4 Serum insulin

Principle

Serum insulin was measured using an Ultrasensitive Rat Insulin ELISA kit (Mercodia, Uppasala, Sweden). The technique was based on the use of two monoclonal antibodies (Abs) that are directed against separate sites on the insulin molecule. During the incubation period, insulin in the rat serum reacted with peroxidase-conjugate and anti-insulin antibodies present in the microtitration well. Subsequently, the plate was subjected to several washing steps which removed unbound enzyme labelled antibody. The bound conjugates were then detected using 3,3',5,5'-tetramethylbenzidine (TMB). Addition of acid stopped the reaction and a colorimetric endpoint was read at 450 nm spectrophotometrically.

Procedure

A serum insulin standard curve was developed using a range of calibrators. A volume 50 μl of each calibrator or sample was added in separate wells, and this was followed by addition of an enzyme conjugate to each well. The plate was then incubated for 2 hours, on a shaker set at 700-900 rpm, at room temperature. Subsequently, the solutions were aspirated and this was followed by six wash steps with 350 μl of 1x wash buffer. Finally, the plate was inverted and then tapped against paper towel. Subsequently, a 200 μl substrate TMB was added to each well and the plate was incubated for 30 minutes to allow for blue colour to develop. Thereafter, a 50 μl of the stop solution (0.5 M H_2SO_4)

was added to each well. The absorbance was then read at 450 nm using a multi-plate reader with 5 sec prior shaking for adequate mixing (Biorad-680, BIORAD Ltd., Japan).

2.7 Histopathological examination of the tissue samples

2.7.1 Sample tissue processing and sectioning

After fixation in 10% phosphate buffered formalin the samples were dehydrated as follows; 70% ethanol for 24 hours, 90% ethanol for 2 hours and 100% for 1 hour, at room temperature. After dehydration, the samples were stored in xylene for 1 hour at room temperature in order to remove the ethanol from the tissue. Thereafter, the tissue samples were submerged in wax at 58 °C overnight in order to allow penetration of the wax into the tissue. The tissues were then placed in embedding molds, arranged into the desired positions and then embedding cassettes were placed on top. The embedding molds were then placed in a cold plate to allow for the wax to solidify. The samples were then stored at room temperature until sectioning. All samples were sectioned at 4 µm, using a microtome, and then attached on normal slides.

2.7.2 Slide staining and viewing

The slides were stained with haematoxylin and eosin, and then scanned with a Slide Scanner Leica SCN400 (Leica Microsystems, Wetzlar, Germany). All snap shots presented in the results section were taken at 400X total magnification.

2.8 Statistical Analysis

All data are expressed as mean ± SD of 5-7 rats. The data were analyzed with a statistical software package (IBM SPSS statistics, version 21), using paired *t-test* separately for the two groups of normal (normal control versus respective toxicological group) and two groups of diabetic rats (diabetic control versus diabetic treated) for chapters 3-6, whilst Tukey's HSD multiple range *post-hoc* test was used for chapter 7. The values were considered statistically different at $p<0.05$. *(asteric)

and #(hash) signs were used to show significance of differences between two groups of normal and two groups of diabetic rats, respectively in chapters 3-6; whilst ^{abc} letters were used for chapter 7.

CHAPTER THREE

Commercially available sucralose-containing sweetener worsens lipid profile, inflammatory biomarkers and organ histopathology in normal and type 2 diabetes model of rats

Siphiwe Ndumiso Dlamini, Nondumiso Prosperity Mbambo, Md. Shahidul Islam*

Biomedical Research Lab, Department of Biochemistry, University of KwaZulu-Natal (Westville Campus), Durban 4000, South Africa

***Corresponding author:**

Dr MS Islam, Department of Biochemistry, School of Life Sciences, University of KwaZulu-Natal (Westville Campus), Durban 4000, South Africa

TEL: +27 31 260 8717; FAX: +27 31 260 7942; E-mail: islAMD@ukzn.ac.za

Running title:

Effects of sucralose on diabetes

Research highlights:

- I. Effects of sucralose-containing sweetener have been investigated in non-diabetic and in a type 2 diabetes model of rats
- II. Sucralose consumption worsened lipid profile and some inflammatory biomarkers in both conditions
- III. Consumption of sucralose damaged brain, liver, heart, kidney and pancreatic tissues in both conditions

3.1 Abstract

The present study investigated the effects of commercially available sucralose both in non-diabetic and type 2 diabetes (T2D) rat model. Seven-week-old male Sprague-Dawley rats were randomly divided into four groups, namely: Normal Control (NC), Toxicological Sucralose (TSCL) Diabetic Control (DBC) and Diabetic Sucralose (DSCL). T2D was induced experimentally in the DBC and DSCL groups only and rats with blood glucose concentrations >300 mg/dl were considered as diabetic. During the 13 week experimental period, the two control groups were administered with normal drinking water, whilst TSCL and DSCL groups received sucralose-containing NNS solutions, *ad libitum*, at concentrations equivalent to the sweetness of 20% and 10% sucrose solution, respectively. Administration of sucralose increased ($p < 0.05$) fluid intake and serum LDL-cholesterol, decreased serum HDL-cholesterol and fructosamine, and caused histological changes in normal rats whilst consumption of sucralose significantly ($p < 0.05$) increased serum cholesterol and alkaline phosphatase, and deteriorated organ tissue histopathology in type-2 diabetic rats. The results of this study indicate that uncontrolled consumption of commercially available sucralose containing NNSs may worsen lipid profile, some inflammatory biomarkers and organ tissue histopathology both in normal and diabetic conditions. Thus, it is recommended that individuals consuming sucralose limit their daily intake.

3.2 Introduction

Diabetes mellitus (DM) is the most common endocrine disorder and affects more than 382 million people globally (IDF Atlas, 2013). There are two major forms of diabetes, type-1 and type-2. The type-2 accounts for 90-95% of all diabetes cases and is characterized by chronic hyperglycaemia, as well as insulin resistance and β -cell dysfunction (Nyenwe et al., 2011). Chronic hyperglycaemia in type-2 diabetes (T2D) is known to cause disorders in the metabolism of carbohydrates, lipids and proteins, ultimately leading to diabetic vascular complications (Abou-Seif et al., 2004). Long-term vascular complications are the major cause of mortality and morbidity in type-2 diabetic individuals; and include retinopathy, neuropathy, nephropathy and cardiovascular disease. Management of T2D

requires lifestyle changes such as healthy diet and physical exercise in order to prevent these long-term complications (Ripsin et al., 2009). Moreover, several studies have clearly demonstrated that reducing calorie intake in the diet is highly effective in obtaining glycaemic control (Ajala et al., 2013; Kirk et al., 2008). Thus, individuals with this form of diabetes are increasingly substituting high calorie-sweeteners such as sucrose with non-nutritive sweeteners (NNSs), when sucralose is widely used in this regard.

Sucralose is a NNS synthesized by selectively substituting the hydroxyl groups of sucrose with chlorine, to produce a molecule that is approximately 600 times sweeter than sucrose (Binns, 2003). This NNS was approved by the Food and Drug Administration in 1998 at an acceptable daily intake (ADI) of 5 mg per kilogram body weight (FDA, 1998). Sucralose has become highly popular as a sugar substitute because of its favourable taste like sucrose and stability at higher temperatures and lower pH, when compared to other NNSs (Binns, 2003). Although both clinical and animal studies have reported that sucralose is poorly absorbed in the small intestine, there is no evidence that this NNS is not metabolized by humans. (Roberts et al., 2000; Wood et al., 2000; John et al., 2000a; Johan et al., 2000b; Sims et al., 2000). Another recent study reported that sucralose sweetener was capable to alter the activity percentage of the radiopharmaceuticals in different organs of rats (Rocha et al., 2011).

Although a number of animal and clinical trials have been conducted to investigate the safety of sucralose for human consumption, the results of these studies are conflicting. Some studies have demonstrated that sucralose is a biologically inert compound and does not affect appetite, glucose homeostasis, glucagon-like protein-1 (GLP-1), and serum insulin (Brown et al., 2011; Ford et al., 2011; Grotz et al., 2003). Other studies suggest that sucralose consumption alters serum glucose, insulin, GLP-1 and lipid profile (Schiffman and Rother, 2013; Saada et al., 2013). Additionally, in most studies sucralose has been used in its pure form, and not in the form in which it is used by people as a sugar substitute. Apart from sucralose, lactose, microcrystalline cellulose, flavours and cross linked sodium carboxymethyl cellulose are present in the commercially available sucralose-containing NNSs with calorie content about 342 kcal per 100 g of sweetener (Canderel website, 2014) which may have different effects compared to pure sucralose. Although Saada et al. (2013) recently

used sucralose in the form in which it is available commercially, to assess the biological effects in diabetic rats; their investigation was different from the present study. The objective of their study was to evaluate the impact of sucralose on oxidative stress in the brain and testis, in addition to the effect on blood glucose, serum insulin and lipid profile using an STZ-induced rat model, which resembles type-1 diabetes. However, many other different organ specific inflammatory biomarkers and organ tissue histopathology were not conducted in their study. Regular assessment of inflammatory biomarkers is extremely important in the monitoring of the progression of diabetes in patients. Hence, the aim of the present study was to determine the effects of commercially available sucralose on diabetes related parameters, some organ specific inflammatory biomarkers and organ tissue histopathology in an experimentally induced rat model of T2D along with normal rat to examine if there any toxicological effects.

3.3 Materials and methods

Please refer to chapter 2 of this thesis (pages 30-39) for details.

3.4 Results

3.4.1 Calculation of the human equivalent dose of sucralose

According to the data presented in the Table 3.1, the concentrations and corresponding consumption of sucralose by the TSCL (20%) and DSCL (10%) groups were 12.41 and 24.82 mg/dl and 11.2 and 18.66 mg/rat/day respectively. However, according to the FDA recommended ADI of sucralose (5 mg/kg bw), the calculated human equivalent dose (ADI) for rats was 30.86 mg/kg bw while the actual sucralose consumption for TSCL group was slightly lower (25.88 mg/kg bw) and for DSCL group was higher (68.82 mg/kg bw) compared to the human equivalent dose (ADI) (Table 3.1).

Table 3.1: Calculation of the human equivalent dosages and actual consumption of sucralose by normal and diabetic rats via drinking water

Animal groups	Mean fluid intake (ml/rat/day)	Mean body weight (g)	Concentration of sucralose (mg/dl)	Consumption of sucralose (mg/rat/day)	Human ADI equivalent (mg/kg bw) ¹	Actual consumption (mg/kg bw) ²
TSCL	45.32	434.29	24.82	11.24	30.86	25.88
DSCL	150.38	271.11	12.41	18.66	30.86	68.82

Data are presented as mean of 5-6 rats.

¹ Human dose (mg/kg bw) = [Animal dose (mg/kg bw) x [Animal K_m / Human K_m]] , where human dose (ADI) is 5 mg/kg bw and K_m is 37 and rats K_m is 6. K_m is a correction factor represents the relation bewtween body weight and body surface area (Natural Health Research Institute).

²Actual consumption (mg/kg bw) = [Consumption of sucralose (ml/rat/day) x 1000] / Mean body weight (g).

ADI, Acceptable Daily Intake; TSCL, Toxicological Sucralose; DSCL, Diabetic Sucralose.

3.4.2 Food and fluid intake, 3h-FBG and OGTT

Administration of commercially available sucralose-containing sweetener to normal rats (TSCL) provoked a significant increase in fluid intake (27%) with no significant effect on food intake and body weight gain when no significant difference was observed for diabetic rats regarding above-mentioned parameters (Fig. 3.1). Although no significant effects were recorded for weekly 3h-FBG and OGTT, when compared to respective values of the control groups or normal and diabetic rats that received normal drinking water (Fig. 3.2 and Fig. 3.3), sucralose treatment resulted in a significant decrease (-28%) in serum fructosamine and corresponding insulin concentration in normal but not in diabetic rats (Table 3.2).

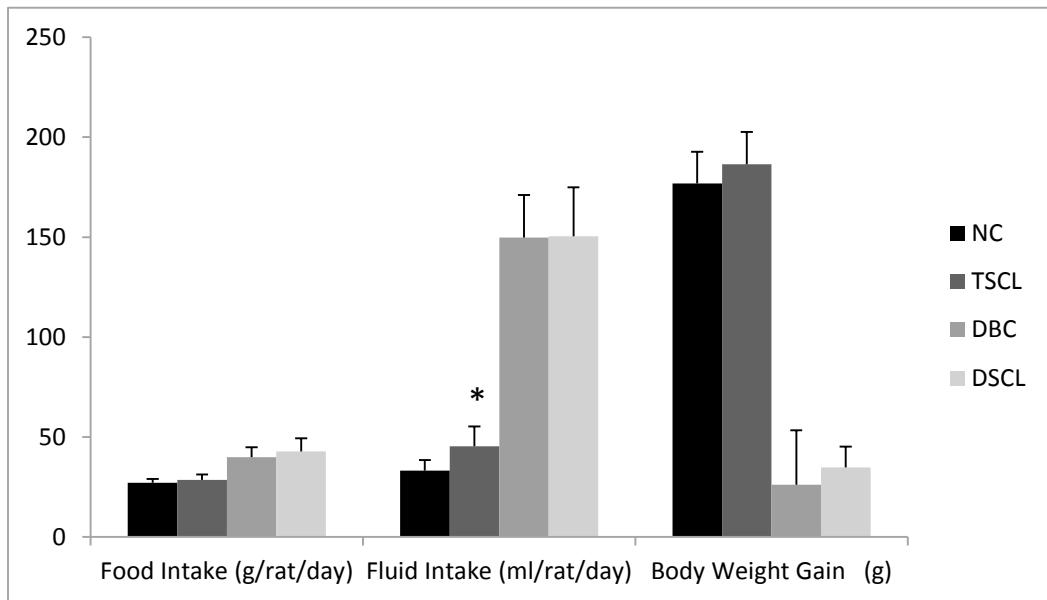


Figure 3.1: Mean food and fluid intake and body weight gain during the entire experimental period. Data presented as mean \pm SD of 5-6 rats. * Significantly different compared to NC group, (Paired *t*-test, $p < 0.05$). NC, Normal Control; TSCL, Toxicological Sucralose; DBC, Diabetic Control; DSCL, Diabetic Sucralose.

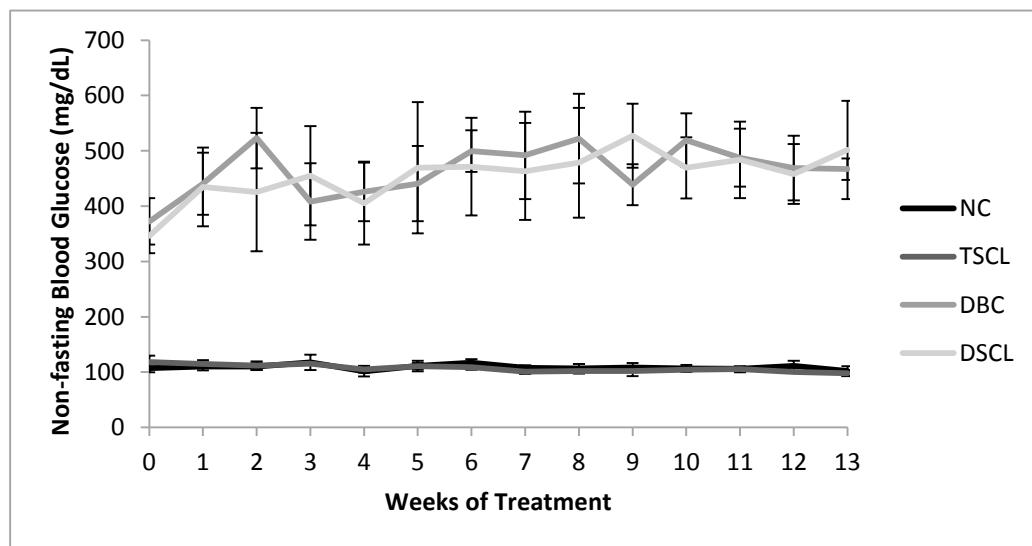


Figure 3.2: Mean weekly non-fasting blood glucose in different rat groups over the 13 week experimental period. Data are presented as mean \pm SD of 5-6 rats. NC, Normal Control; TSCL, Toxicological Sucralose; DBC, Diabetic Control; DSCL, Diabetic Sucralose.

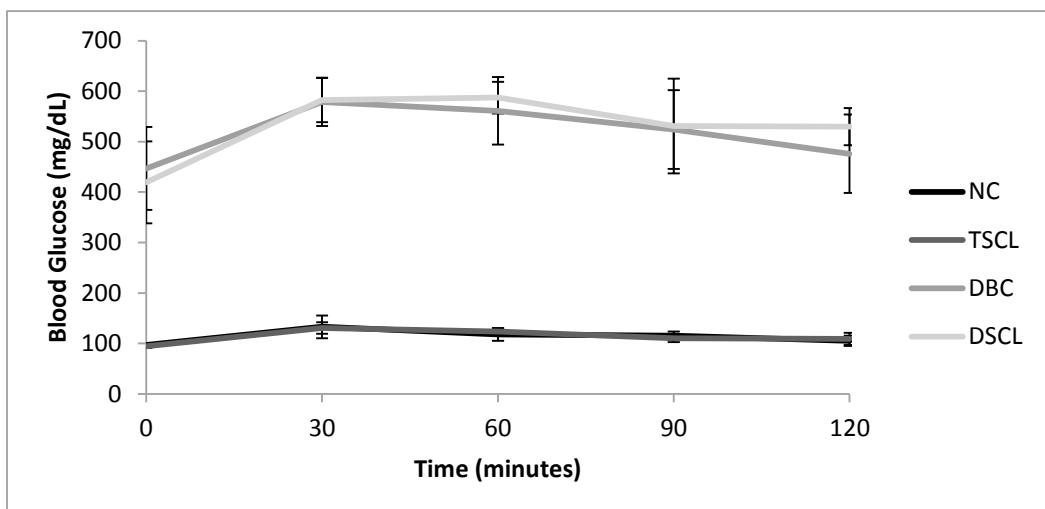


Figure 3.3: Oral glucose tolerance test (OGTT) in different animal groups at week 12 of the 13 week experimental period. Data are presented as mean \pm SD of 5-6 rats. NC, Normal Control; TSCL, Toxicological Sucralose; DBC, Diabetic Control; DSCL, Diabetic Sucralose.

3.4.3 Serum lipid profile

The data of serum lipid profile are presented in Fig. 3.4. The sucralose treatment significantly increased serum LDL-cholesterol (71%) and decreased serum HDL-cholesterol (-34%) in normal rats and increased total cholesterol in diabetic rats (42%) compared to their respective controls. Triglyceride concentration was not affected by sucralose treatment neither in normal nor in diabetic rats (Fig. 3.4).

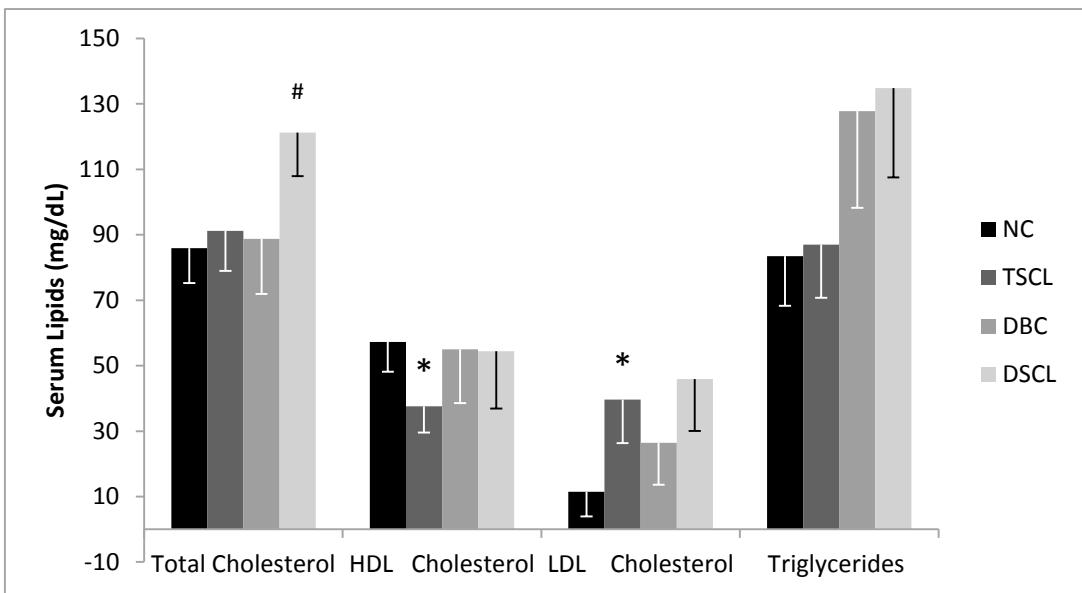


Figure 3.4: Serum lipid profile in the different animal groups at the end of the experimental period. Data are presented as mean \pm SD of 5-6 rats. * and # Significantly different compared to NC and DBC groups, respectively (Paired *t*-test, $p < 0.05$). NC, Normal Control; TSCL, Toxicological Sucralose; DBC, Diabetic Control; DSCL, Diabetic Sucralose; HDL, High Density Lipoproteins, LDL, Low Density Lipoproteins.

3.4.3 Serum ALP and liver function enzymes

The data for serum alkaline phosphatase (ALP) and two major liver function enzymes, alanine transaminase (ALT) and aspartate transaminase (AST), are presented in Fig. 3.5. Data shows that feeding of sucralose significantly and drastically increased the level of ALP in the diabetic rats but not in normal rats. Although the concentration of ALT was significantly increased in the diabetic rats compared to normal rats, sucralose did not affect the concentrations of ALT and AST both in normal and diabetic rats.

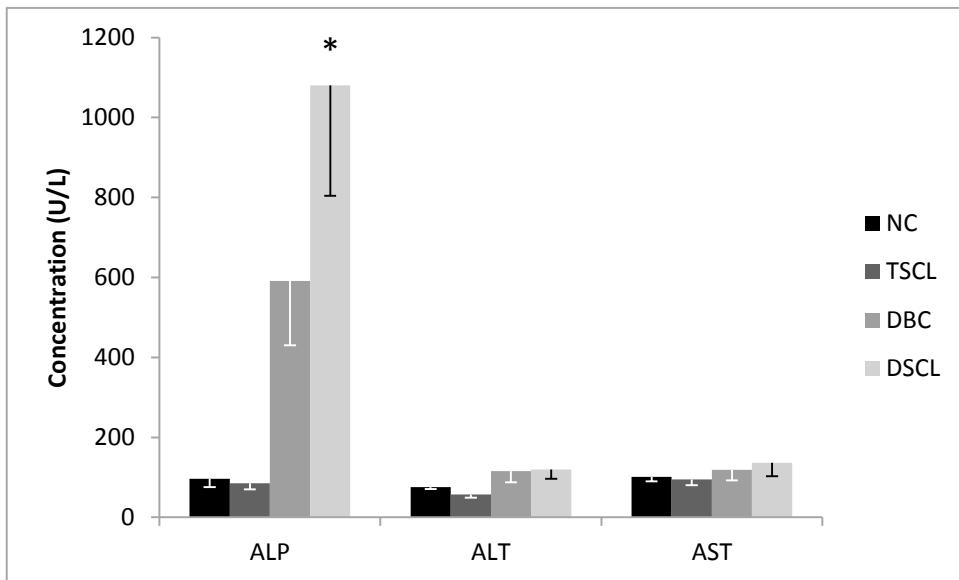


Figure 3.5: Concentrations of alkaline phosphates and liver function enzymes in the serum of different animal groups at the end of the experimental period. Data are presented as mean \pm SD of 5-6 rats.* Significantly different compared to NC groups (Paired *t*-test, $p < 0.05$). NC, Normal Control; TSCL, Toxicological Sucralose; DBC, Diabetic Control; DSCL, Diabetic Sucralose; ALP, Alkaline phosphatase; ALT, Alanine transaminase; AST, Aspartate transaminase.

3.4.4 Serum insulin, fructosamine, LDH, CK-MB, uric acid and creatinine

Table 3.2 shows the concentrations of serum insulin, fructosamine, lactate dehydrogenase (LDH), Creatine kinase (CK-MB), uric acid and creatinine in the different group of rats. Although serum insulin and corresponding serum fructosamine concentrations were decreased in the sucralose consuming normal and diabetic rats compared to their corresponding controls, the significant reduction was only observed in TSCL group ($p < 0.05$) and nearly significant in the DSCL group ($p = 0.071$) (Table 3.2). Serum CK-MB and LDH concentrations were significantly increased in the sucralose consuming diabetic rats (DSCL group) compared to the DBC group when no significant difference was observed for normal rats (Table 3.2). Consumption of sucralose did not significantly affect the concentration of serum creatinine either in normal or diabetic rats (Table 3.2).

Table 3.2: Serum LDH, uric acid, fructosamine, CK-MB, creatinine and insulin at the end of the experimental period

	NC	TSCL	DBC	DSCL
Insulin (ng/L)	152.51 ± 56.18	115.91 ± 56.91	74.74 ± 8.08	67.71 ± 14.03
Fructosamine (μmol/L)	505.40 ± 31.39	365.20 ± 19.43 *	588.80 ± 30.34	555.00 ± 20.03
LDH (U/L)	1129.00 ± 295.00	1084.25 ± 467.91	2270.5 ± 1183.71	3085.00 ± 1080.75
CK-MB (U/L)	1331.98 ± 669.40	1196.55 ± 415.41	1926.52 ± 587.81	3007.58 ± 627.79
Uric acid (mg/dL)	9.44 ± 3.46	7.84 ± 1.69	9.74 ± 0.98	12.47 ± 1.79
Creatinine (mg/dL)	4.73 ± 0.87	3.63 ± 1.08	3.89 ± 0.87	3.91 ± 0.62

Data presented as mean ± SD of 5-6 rats. * Significantly different compared to NC groups (Paired *t*-test, *p* < 0.05). NC, Normal Control; TSCL, Toxicological Sucralose; DBC, Diabetic Control; DSCL, Diabetic Sucralose; LDH, Lactate dehydrogenase; CK-MB, Creatine kinase (heart specific).

3.4.5 Histopathological findings

Results from the histopathological examination are shown in Fig. 3.6. It is observed that diabetes caused noticeable changes to the tissues of the liver, heart, kidney and pancreas. The histology of the brain did not seem to be affected by diabetes. In comparison to the normal groups, the pancreases in the two diabetic groups (Figure 3.6.19 & 20) appeared to have degenerative changes and reduced cellularity in the islets of Langerhans, and the consumption of sucralose appeared to enhance the damage of the islets both in normal and diabetic rats (Figure 3.6.18 & 20). The consumption of high dose sucralose appears to lead to severe degenerative changes in the brain tissue of normal rats (Figure 3.6.2) when opposite results were observed in the diabetic rats (Figure 3.6.4) fed a lower dose of sucralose. Hepatic degenerations also appeared to be improved by the consumption sucralose both in normal and diabetic rats (Figure 3.6.6 & 8). The kidneys of the two diabetic groups (Figure 3.6.15 & 16) showed morphological changes in the glomeruli when compared to the normal groups (Figure 3.6.13 & 14). Administration of sucralose for 13 week had apparent effect on the histology of the kidney tissues in both the diabetic and normal rats (Figure 3.6.14 & 16) and the consumption of sucralose may cause slight degeneration of cardiac tissues both in normal (Figure 3.6.10) and diabetic rats (Figure 3.6.12) compared to their corresponding controls (Figure 3.6.9 & 11).

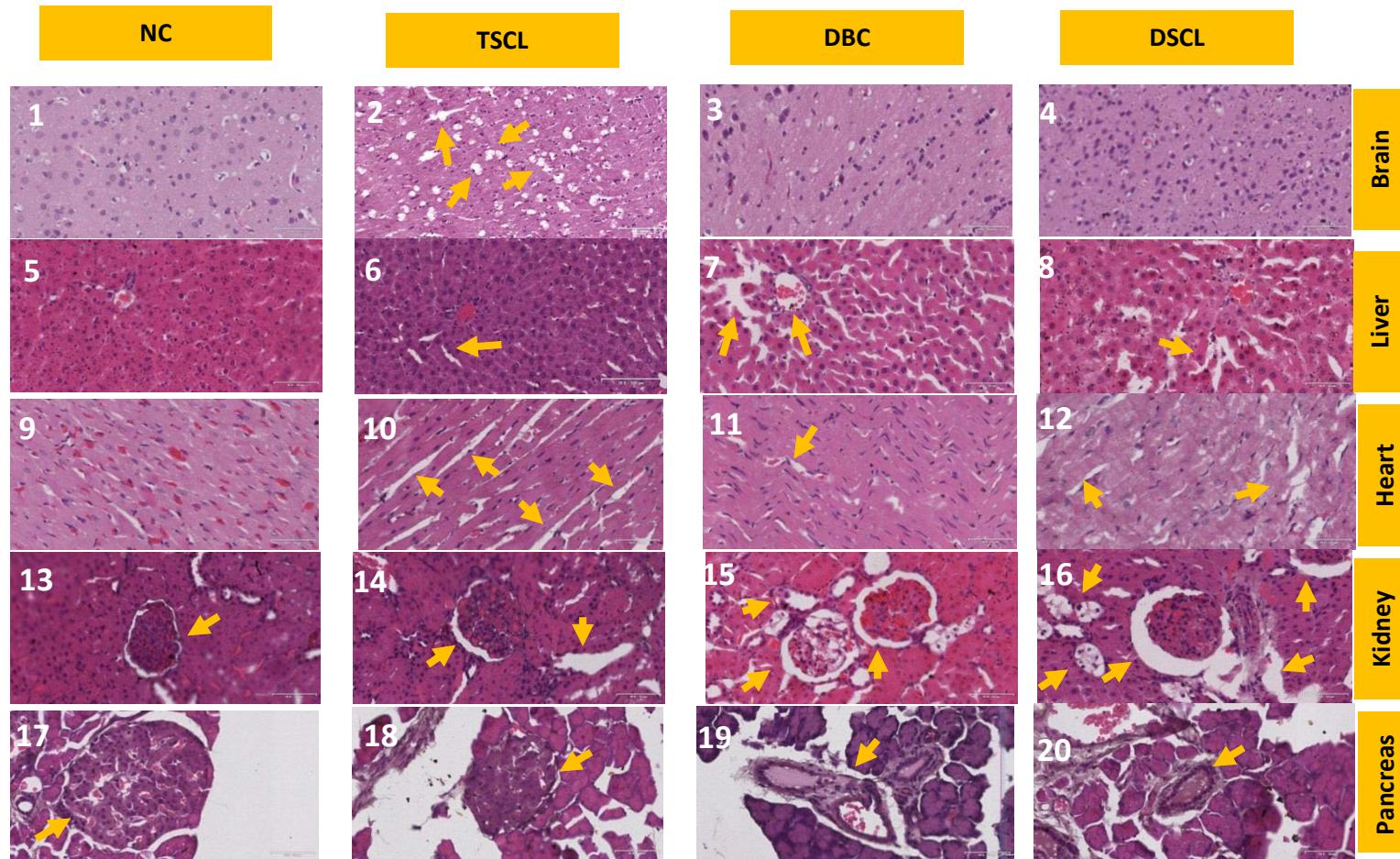


Figure 3.6: Histological examinations of the brain (1-4), liver (5-8), heart (9-12), kidney (13-16) and pancreas (17-20) of different animal groups at the end of the experimental period; at X400 total magnification. NC, Normal Control; TSCL, Toxicological Sucralose; DBC, Diabetic Control; DSCL, Diabetic Sucralose.

3.5 Discussion

Sucralose is a popular NNS used in a number of food products as it provides more sweetness than sugar without the undesired calories. Majority of studies have reported contradicting results and have used sucralose in its pure form which is not consumed by people. The present study was conducted to examine the effects of commercially available sucralose-containing sweeteners both in normal and type 2 diabetic rats. Since the sweetener was supplied to rats with drinking water and diabetic rats usually consume significantly higher amount of water compared to normal rats so a lower concentration (10%) was used for diabetic rats compared to the normal rats (20%) to keep their consumption at a similar level. According to the calculation, the normal rats consumed slightly less and diabetic rats consumed bit higher amount of sucralose compared to the FDA recommended human equivalent dose due to the higher consumption of water (Table 3.1).

The administration of commercially available sucralose ad libitum for 13 week in normal rats increased fluid intake (Figure 3.1) and serum LDL-cholesterol, decreased serum HDL-cholesterol (Figure 3.4) and fructosamine (Table 3.2), and caused histological changes in the brain, liver, heart and pancreas (Figure 3.6). On the other hand, the sucralose consuming diabetic rats presented with increased serum cholesterol (Figure 3.4) and serum alkaline phosphatase (Figure 3.5), lactate dehydrogenase and creatine kinase (Table 3.2) in addition to the damage to the tissue of major metabolic organs including pancreas (Figure 3.6), an organ which is first affected before the early onset of diabetes.

According to the American Diabetes Association (ADA, 2007), common symptoms of diabetes include polyphagia, polydipsia and unexplained weight loss. In this study, the diabetic rats showed increased food and fluid intake, in addition to decreased body weight, compared to the respective values in the normal control group. Additionally, T2D is characterized by chronic hyperglycaemia, as well as insulin resistance and β -cell dysfunction (Nyenwe et al., 2011). In the current study, the diabetic rats exhibited significantly increased weekly 3h-FBG, glucose intolerance and significantly reduced serum insulin concentration, indicative of the major characteristics of T2D. Administration of sucralose to both diabetic and normal rats did not alter food and fluid intake, body weight gain and

glucose homeostasis in the present study. These results corroborate with previous studies which suggest that sucralose consumption may have no effect on glucose homeostasis, body weight gain and energy metabolism, when compared to control groups consuming water (Baird et al., 2000; Brown et al., 2011; Grotz et al., 2003). Nevertheless, conflicting to our results, Saada et al. (2013) recently reported that sucralose administration at a dose of 11 mg/kg body weight in diabetic rats resulted in a significant decrease in serum glucose concentrations after 6 weeks. The differences in the effect on blood glucose in their investigation and the current study may be attributable to different animal models, experimental designs and type of commercially available sucralose used. In their study, sucralose was used as Splenda containing glucose and maltodextrin, in addition to 1.10% sucralose which was administered with as a single oral dose once daily to type-1 diabetic rats; whereas in the present study, Canderel yellow was used and contains lactose, microcrystalline cellulose and carboxy methyl cellulose, in addition to 7.3% sucralose and was administered with to normal or type-2 diabetic rats ad libitum with drinking water at an equivalent dose of the sweetness of 10% sugar which is equivalent to 0.0126% sucralose solution. Hence, due to the very lower dose of sucralose used in our study it was not effective in reducing blood glucose concentrations.

It is well established that dyslipidaemia is one of the major metabolic complications of diabetes (Mooradian, 2009). In the present study, induction of T2D led to significantly increased serum triglycerides, without affecting serum total cholesterol, HDL cholesterol and LDL cholesterol, compared to respective values in the normal control group. During T2D, insulin resistance in the adipose tissue leads to defective storage of fatty acids. Thus, more fatty acids are channelled into the liver and disturb insulin signalling, ultimately increasing de novo lipogenesis by activating key transcription factors, such as SREBP1-c (Matikainen and Taskinen, 2012). Thus, it can be observed that sucralose consumption induces and worsens dyslipidaemia in normal and type 2 diabetic rats, respectively. The findings of the current study are in accordance with the recent study by Saada et al. (2013), which suggested that sucralose administration to diabetic rats alters the serum lipid profile. Elevated total cholesterol in the serum of diabetic rats treated with sucralose may have resulted from increased absorption in the small intestine and increased de novo synthesis. Increased serum LDL-cholesterol due to administration of sucralose in both diabetic and normal rats might be caused by

elevated lipolysis of very low density lipoproteins (VLDL), precursors for LDL production, by lipoprotein lipase. In contrast to our results, some studies have demonstrated that sucralose consumption does not alter total cholesterol in human subjects (Baird et al., 2000). The difference in the findings might be not only due to the different experimental design and dose but also due to the different metabolic patterns of sucralose in different species. Further studies are needed to confirm this hypothesis.

Abnormalities in liver function tests are more common in type 2 diabetic patients than non-diabetic individuals, and usually indicate liver tissue damage. According to Harris (2005), the excess fatty acids found in the liver during an insulin resistance state induce direct hepatocyte damage. The mechanism by which hepatocytes are damaged is not well established but a number of theories have proposed and these include: disruption to the cell membrane, dysfunction of the mitochondria, formation of toxins and disruption of key steps involved in regulation of metabolism (Neuschwander-Tetri and Caldwell, 2003). In our study, significantly increased ALT and ALP concentrations in the diabetic groups (Figure 3.5) were consistent with the histopathological findings (Fig. 3.6) which showed liver and some other organ tissue damage as a result of diabetes.

Lactate dehydrogenase (LDH) and ALP are enzymes extensively expressed in body tissues and are thus released to the blood during tissue damage. Studies on the influence of diabetes in serum LDH have reported conflicting results. A number of investigations, including the present study, have suggested that serum LDH is not affected by diabetes (Oliver et al., 1993). On the other hand, other studies have indicated that LDH is significantly increased as a result of diabetes. A study by Celik et al. (2002) revealed that LDH is elevated in alloxan-induced diabetic rabbits. Theories that have been proposed to explain elevated LDH include: insulin resistance in tissues liver and muscle tissue, infection, hepatic anoxia and malnutrition (Celik et al., 2002). Although sucralose consumption did not influence serum LDH significantly in diabetic rats, elevated ALP was indicative of tissue damage in the diabetic rats. The toxicological effect of this commercially available sweetener in the different tissues of both normal and diabetic rats might be due to bioaccumulation of this NNS in these organs. Sucralose is an organochlorine compound, and it has been reported that organochlorines bioaccumulate in tissues of animals (Aronson et al., 2000; Weisbrod et al., 2001). According to

Schiffman and Rother (2013), sucralose is a lipophilic and hydrophilic compound and thus is potentially bioaccumulative. Therefore, it is possible that sucralose or its metabolites accumulates in the organs to cause tissue damage. Further studies are required to confirm this hypothesis.

3.6 Conclusions

It can be concluded from the results obtained from the present study that uncontrolled consumption of commercially available sucralose increases fluid intake and serum alkaline phosphatase while worsening lipid profile and morphology of metabolic organs in normal and diabetic rats. Thus, it is recommended that daily consumption of sucralose must be limited under both normal and diabetic conditions.

3.7 References

Please refer to the reference section at the end of this thesis (pages 126-144).

CHAPTER FOUR

**Commercially available saccharin-containing sweeteners may not be
suitable for patients with type 2 diabetes**

Siphiwe Ndumiso Dlamini, Nondumiso Prosperity Mbambo, Md. Shahidul Islam*

Biomedical Research Lab, Department of Biochemistry, University of KwaZulu-Natal (Westville Campus), Durban 4000, South Africa

***Corresponding author:**

Dr MS Islam, Department of Biochemistry, School of Life Sciences, University of KwaZulu-Natal (Westville Campus), Durban 4000, South Africa

TEL: +27 31 260 8717; FAX: +27 31 260 7942; E-mail: islAMD@ukzn.ac.za

Running title:

Effects of saccharin on diabetes

4.1 Abstract

Non-nutritive sweeteners (NNSs) such as saccharin are becoming more popular as sugar substitutes for diabetic patients. Although saccharin has been extensively studied, majority of studies have reported contradictory results and have used saccharin in its pure form. In the present study, commercially available saccharin is used to assess its effects in normal and type 2 diabetes (T2D) model of rats. Seven-week-old male Sprague-Dawley rats were randomly divided into four groups, as follows: Normal Control (NC), Toxicological Saccharin (TSAC), Diabetic Control (DBC) and Diabetic Saccharin (DSAC). T2D was induced in the DBC and DSAC groups only and rats with 3 hour fasting blood glucose (3h-FBG) concentrations ≥ 300 mg/dl were considered as diabetic. During 13 week experimental period, control groups were administered with normal drinking water, when TSAC and DSAC groups were receiving saccharin-containing NNS solutions, *ad libitum*, at concentrations equivalent to the sweetness of 20% and 10% sucrose solution, respectively. Administration of 20% saccharin to normal rats; increased fluid intake, serum lactate dehydrogenase and reduced serum fructosamine and most lipid concentrations and did not affect the histology in normal rats. Similarly, administration of 10% saccharin to diabetic rats; reduced fructosamine and serum lipids but increased serum alkaline phosphatase and worsened the tissue morphology of liver, kidney and pancreas in diabetic rats. These results indicate that although saccharin-containing NNS may be beneficial for normal humans, it may not be suitable for the people with T2D. Further clinical study is needed to confirm this hypothesis.

4.2 Introduction

Diabetes Mellitus is a global health problem and affects more than 382 million people worldwide (IDF Atlas, 2013). There are two major forms; type 1 and type 2, when type 2 diabetes (T2D) contributes about 90-95% of all diabetes cases. T2D is basically characterized by the progressive decline in insulin action or insulin resistance and reduced ability of β -cell to compensate for insulin resistance or partial β -cell dysfunction (ADA, 2013). Chronic hyperglycemia leads to metabolic changes of lipids, carbohydrates, and proteins; ultimately causing vascular complications such as

diabetic neuropathy, retinopathy, nephropathy and cardiovascular disease (Abou-Seif et al., 2004). Consumption of sucrose sweetened food and drinks may worsen the concentrations as well as the management of blood glucose in type 2 diabetic individuals, as previous studies confirmed that the consumption of sugar, especially in liquid form, increases energy intake and weight gain, visceral adiposity, lipid dysregulation, insulin resistance and the risk of developing T2D (Schulze et al., 2004; Dhingra et al., 2007; Malik et al., 2009; Stanhope et al., 2009; Malik, 2010). Thus diabetic patients are increasingly substituting sucrose with alternative sweeteners in their everyday meals. Although a number of different forms of sweeteners with different sweetness levels and calorie content exist, non-nutritive sweeteners (NNSs) have gained a lot of popularity due to their significantly higher sweetness levels compared to sugar without any undesired calories.

Saccharin is the oldest and most extensively studied NNS in relation to its biological effects as a food additive. This NNS is widely used in many countries due to its stability at high temperatures, long shelf life and low cost (Singh, 2013). Previous studies on the biological effects of saccharin have been largely conflicting and thus the safety of this NNS for human consumption remains debatable (Park et al. 2010; Swithers et al., 2010; Abdelaziz and Ashour 2011; Swithers et al., 2012; Andrejic et al., 2013; Swithers et al., 2013). Abdelaziz and Ashour (2011) reported that supplementation of 35 mg/kg BW of saccharin for 35 days exhibited significantly reduced blood glucose, serum triglycerides and serum cholesterol in female rats. In contrast, a recent study by Andrejic et al., (2013) revealed that 0.0005% saccharin in drinking water, administered to rats for 6 weeks increased blood glucose and body weight but reduced food intake compared to the control, which received normal drinking water. According to the authors, effects on metabolic parameters might be caused by hormonal and neural changes, particularly in the female subgroup since both male and female groups were compared. Another study suggested that 0.1% saccharin solution administered to rats for 3 weeks did not show any significant effect on food intake and weight gain (Park et al., 2010). Additionally, some studies have revealed that 0.3% saccharin-sweetened yogurt given to rats, causes impaired glucose homeostasis, diminished caloric compensation, increased food intake and more elevated weight gain when compared to 20% glucose-sweetened yogurt (Swithers et al., 2012; Swithers et al., 2013).

Hence, from the contradictory data of the above-mentioned studies it is still not clear the actual effects of saccharin consumption either in normal or diabetic condition.

Moreover, majority of these studies have used saccharin in its pure form and not in the form that is consumed by people. Thus, the present study was primarily aimed at assessing the effects of commercially available saccharin-containing NNS on diabetes-related parameters and its associated complications, in an experimentally induced rat model of T2D. The study also simultaneously investigated the toxicological effects of saccharin with a higher dose in non-diabetic rats.

4.3 Materials and methods

Please refer to chapter 2 of this thesis (pages 30-39) for details.

4.4 Results

4.4.1 Daily food and fluid intake, and body weight gain

Administration of a commercially available saccharin-based NNS solution, at a high dose, to normal rats increased fluid intake (51%). Although daily food intake was not affected, the results show that body weight gain after 13 week of treatment was non-significantly elevated (49%) in the diabetic rats treated with a low dose of saccharin (Figure 4.1).

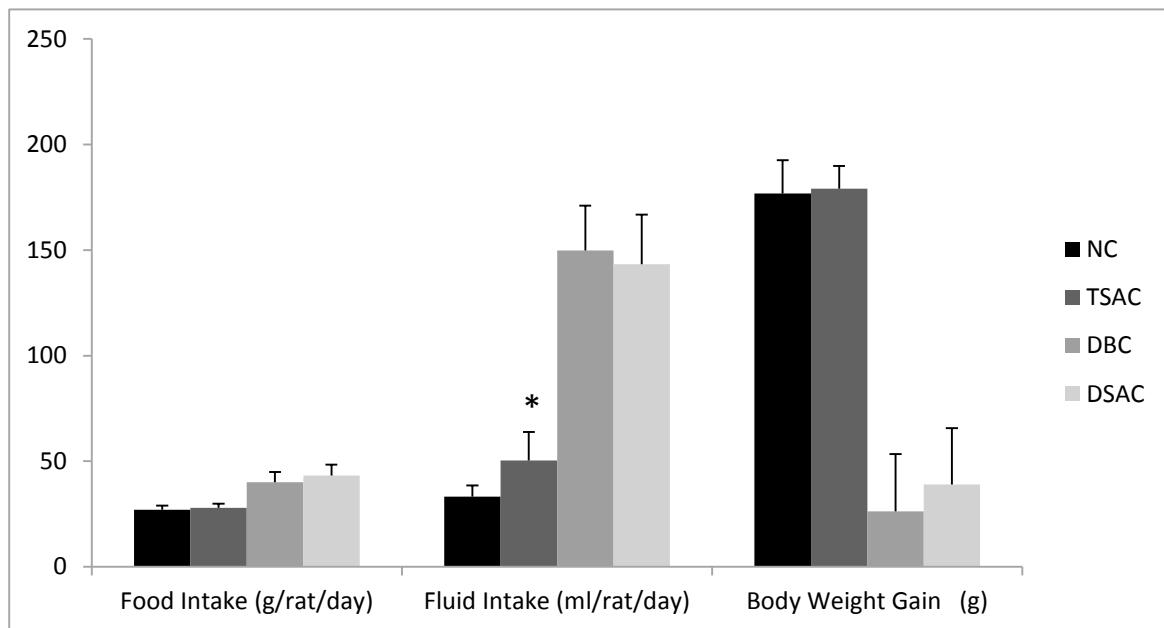


Figure 4.1: Mean food and fluid intake and body weight gain during the entire experimental period. Data presented as mean \pm SD of 5-6 rats. * Significantly different compared to NC group, (Paired *t*-test, $p < 0.05$). NC, Normal Control; TSAC, Toxicological Saccharin; DBC, Diabetic Control; DSAC, Diabetic Saccharin.

4.4.2 Non-fasting blood glucose and oral glucose tolerance test

Administration of saccharin to normal rats caused a significant reduction in serum fructosamine (-26%) and non-significant reduction in serum insulin (-27%); whilst administration to diabetic rats caused a significant reduction in serum fructosamine (-16%), without affecting serum insulin concentrations (Table 4.1).

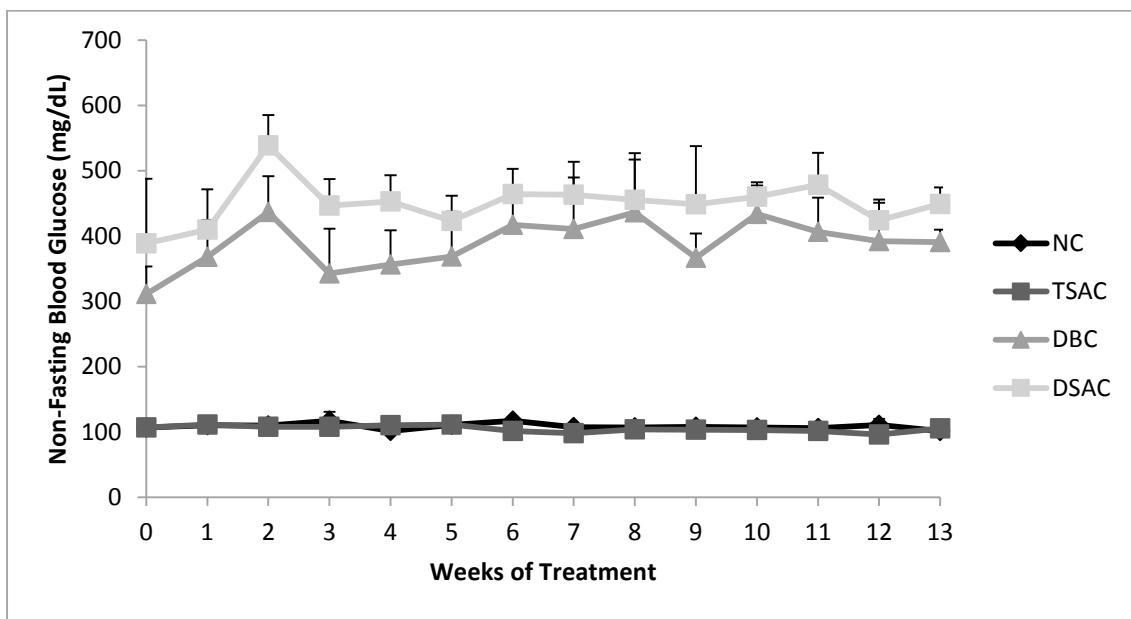


Figure 4.2: Mean weekly non-fasting blood glucose in different rats groups over the 13 week experimental period. Data are presented as mean \pm SD of 5-6 rats. NC, Normal Control; TSAC, Toxicological Saccharin; DBC, Diabetic Control; DSAC, Diabetic Saccharin.

Table 4.1: Serum insulin, fructosamine, ALP, ALT, AST, LDH, CK-MB, uric acid, and creatinine at the end of the experimental period

	NC	TSAC	DBC	DSAC
Insulin (ng/L)	152.51 ± 56.18	111.72 ± 36.29	74.74 ± 8.08	75.50 ± 12.29
Fructosamine (μmol/L)	505.40 ± 31.39	372.20 ± 34.43 *	588.80 ± 30.34	494.83 ± 34.50 #
ALP (U/L)	96.40 ± 20.84	92.25 ± 7.41	591.00 ± 160.53	1195.20 ± 303.67 #
ALT (U/L)	75.80 ± 4.97 a	59.80 ± 5.81 *	115.60 ± 28.40	129.67 ± 15.00
AST (U/L)	101.00 ± 10.93	111.20 ± 19.10	118.80 ± 26.41	115.57 ± 18.47
LDH (U/L)	1129.00 ± 295.00	2118.60 ± 772.67 *	2270.5 ± 1183.71	1162.00 ± 133.62
CK-MB (U/L)	1331.98 ± 669.40	1364.96 ± 414.43	1926.52 ± 587.81	1262.17 ± 251.39
Uric acid (mg/dL)	9.44 ± 3.46	12.46 ± 1.28	9.74 ± 0.98	9.90 ± 3.45
Creatinine (mg/dL)	4.73 ± 0.87	5.36 ± 1.54	3.89 ± 0.87	3.38 ± 0.70

Note: Data presented as mean ± SD of 5-6 rats. * and # Significantly different compared to NC and DBC groups, respectively (Paired *t-test*, $p < 0.05$). NC, Normal Control; TSAC, Toxicological Saccharin; DBC, Diabetic Control; DSAC, Diabetic Saccharin; ALP, Alkaline Phosphatase; ALT, Alanine Transaminase (ALT); AST, Aspartate Transaminase; LDH, Lactate dehydrogenase; CK-MB, Creatine kinase (heart specific).

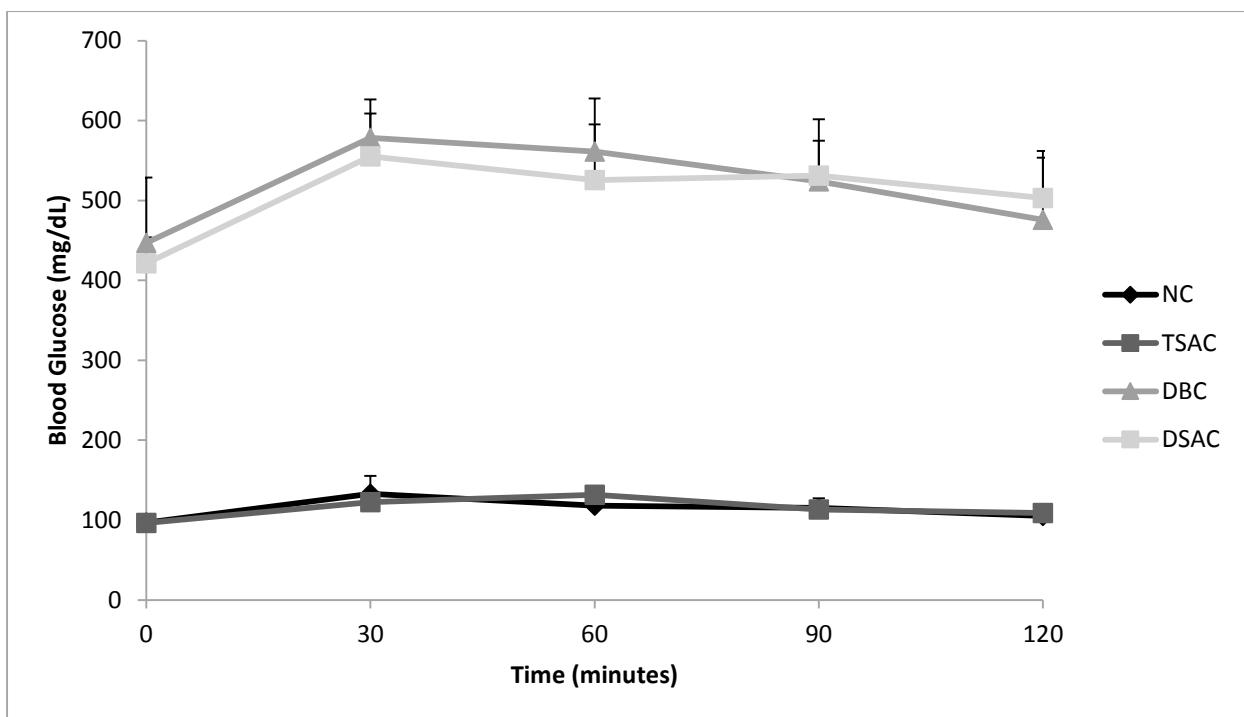


Figure 4.3: Oral glucose tolerance test (OGTT) in different animal groups at week 12 of the 13 week experimental period. Data are presented as mean \pm SD of 5-6 rats. NC, Normal Control; TSAC, Toxicological Saccharin; DBC, Diabetic Control; DSAC, Diabetic Saccharin.

4.4.3 Serum lipid profile

The high dose of saccharin administration for 13 week to normal rats resulted in a significant decrease in serum HDL-cholesterol (-41%) and serum triglycerides (-47%), and a significant increase in serum LDL-cholesterol (113%), without affecting total cholesterol. Administration of a low dose saccharin to diabetic rats non-significantly reduced serum LDL-cholesterol and serum triglycerides, without affecting serum HDL- and total Cholesterol (Figure 4.4) which revealed the beneficial effects saccharin consumption in diabetic condition.

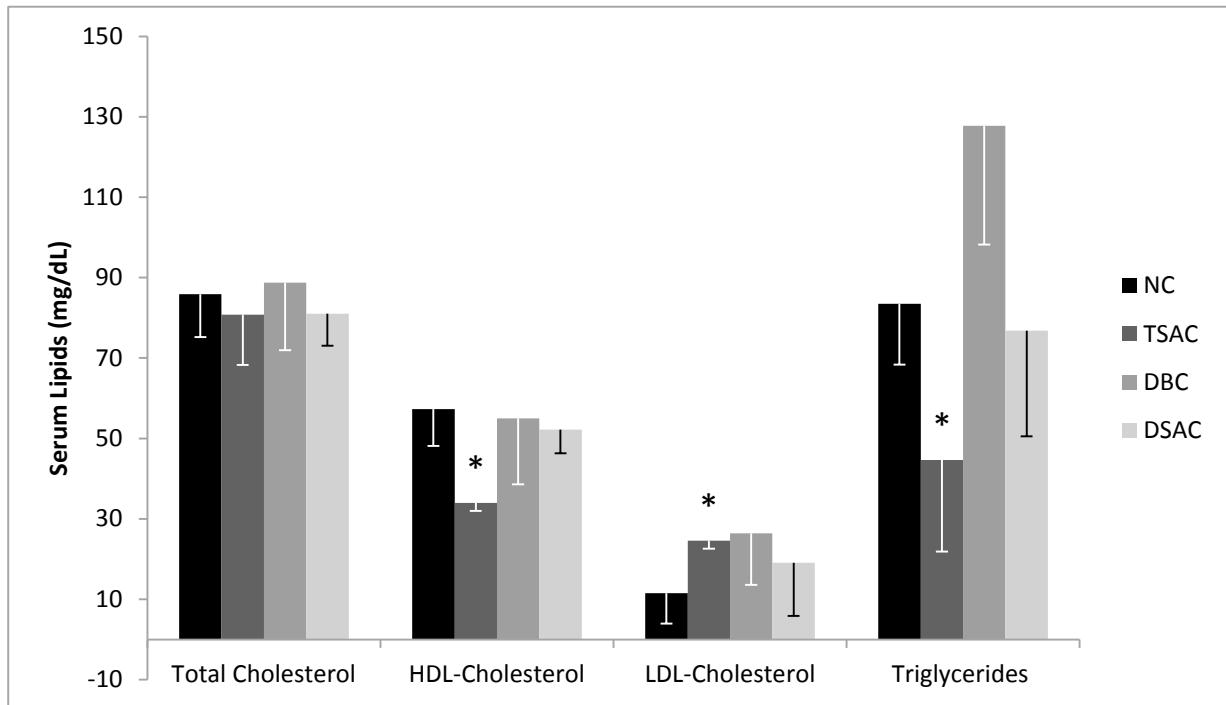


Figure 4.4: Serum lipid profile in the different animal groups at the end of the experimental period. Data are presented as mean \pm SD of 5-6 rats. * Significantly different compared to NC group, (Paired *t*-test, $p < 0.05$). NC, Normal Control; TSAC, Toxicological Saccharin; DBC, Diabetic Control; DSAC, Diabetic Saccharin.

4.4.4 Serum insulin, fructosamine, ALP, ALT, AST, LDH, CK-MB, uric acid, and creatinine

Normal rats treated with a high dose of saccharin exhibited significantly decreased serum alanine transaminase (-21%) (Table 4.1) and significantly increased lactate dehydrogenase (88%) (Table 4.1). On the other hand, diabetic rats treated with a low dose of saccharin showed significantly and drastically increased serum alkaline phosphatase (102%) at the end of the experimental period (Table 4.1). Heart specific creatine kinase (CK-MB), uric acid and creatinine concentrations in the serum were not affected by saccharin treatment (Table 4.1).

4.4.5 Histopathological findings

The histopathological findings are shown in Figure 4.5. Administration of saccharin provoked morphological changes in the tissue of the heart, kidney and pancreas in both normal and diabetic rats. Examination of the heart tissue (Figure 4.5.9-12) revealed that saccharin led to noticeably reduced tissue damage in the heart (yellow arrows), in diabetic rats. After saccharin treatment, in the kidneys, the Bowman's capsule (BC) appeared to be increased and there was vacuolation of the convoluted tubules in control (Figure 4.5.14) and diabetic rats (Figure 4.5.16). Interestingly, saccharin administration also appeared to reduce the islet sizes in control rats (Figure 4.5.18). The histology of the brain and liver appeared normal after saccharin treatment in both control groups (Figure 4.5.2 for the brain and Figure 4.5.6 for the liver).

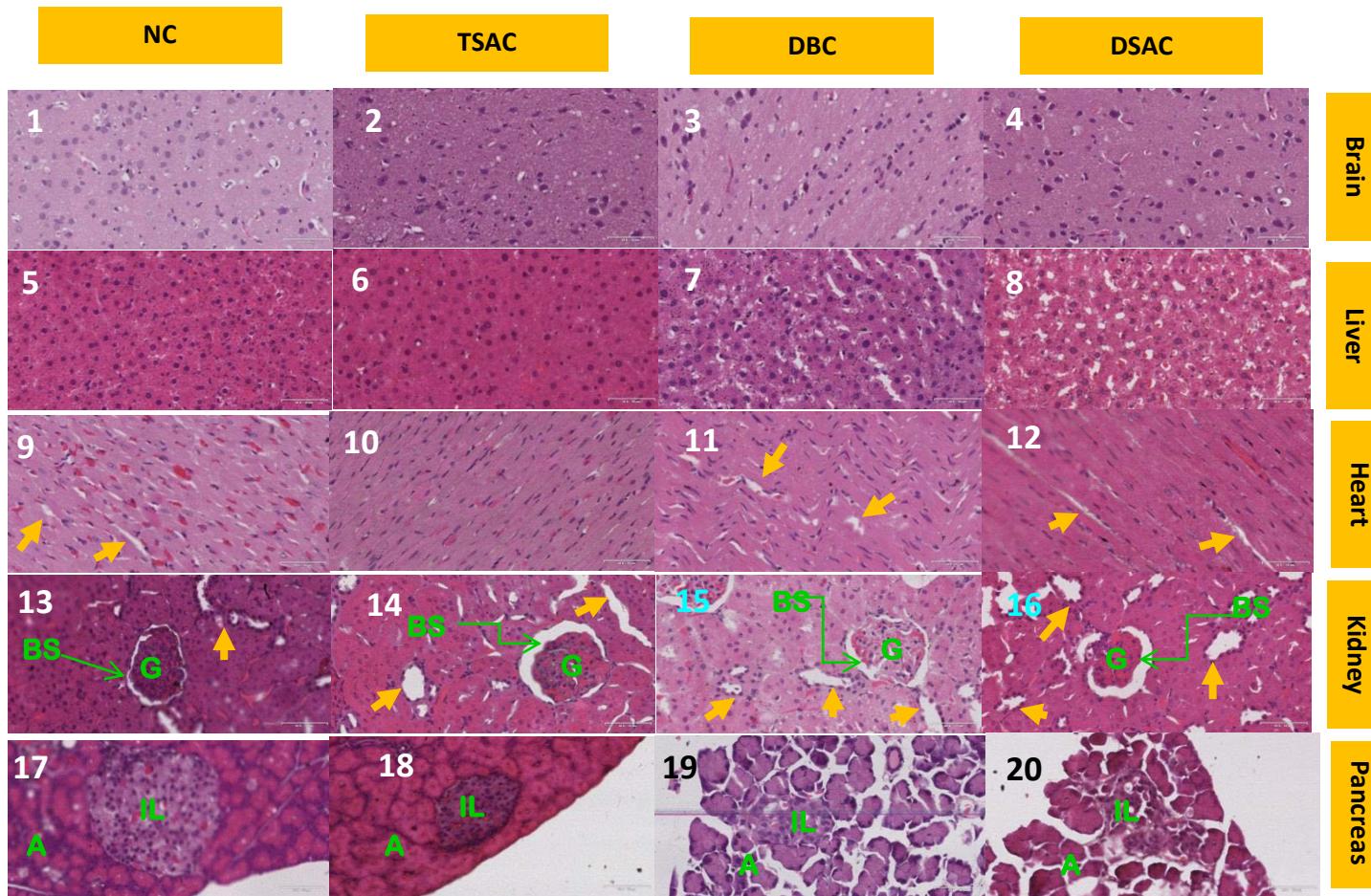


Figure 4.5: Histological examinations of the brain (1-4), liver (5-8), heart (9-12), kidney (13-16) and pancreas (17-20) of different animal groups at the end of the experimental period; at 400x total magnification. NC, Normal Control; TSAC, Toxicological Saccharin; DBC, Diabetic Control; DSAC, Diabetic Saccharin; BS, Bowmans Space; G, Glomerulus; A, Acini; IL, Islets of Langerhans.

4.5 Discussion

Several studies have been conducted to assess the safety of saccharin consumption by humans, when majority of the studies have reported conflicting results and used saccharin in its pure form and not in the commercially available form, which is consumed by people. Thus, the present study was aimed at investigating the pharmacological and toxicological effects of commercially available saccharin-containing NNS in an experimentally induced rat model of T2D and normal rats, respectively. The key findings suggest that saccharin containing NNS may have unfavorable effects on serum lipid profile both in normal and diabetic rats; additionally, significant changes in the kidney and pancreatic tissue histopathology in both normal and diabetic rats and corresponding drastically increased serum alkaline phosphatase (ALP) concentration and increased body weight in diabetic rats raised question about the safety of the saccharin-containing NNS, at least for type 2 diabetic patients. The data of our study are discussed below in more details.

In our study, the diabetic rats exhibited increased food and fluid intake, and decreased weight gain compared to the normal rats. Several studies have demonstrated that saccharin treatment leads to increased weight gain, food intake, decreased GLP-1 release and impaired glucose homeostasis (Swithers et al., 2009; Swithers et al., 2010; Swithers et al., 2012; Feijó et al., 2013), whilst others have reported that saccharin does not affect these parameters (Park et al., 2010; Bryant et al., 2014). The differences in the reported results may be due to different experimental designs that were used. Most studies that reported negative effects on energy metabolism compared saccharin to calorie-containing sugars; either glucose or sucrose. It has been reported that intake of high calories before main meal leads to reduce food intake, a process known as caloric compensation (Allison, 2013). Thus, it is possible that when saccharin consumption led to increased food intake and ultimately elevated weight gain; the results were rather due to decreased food intake in the control groups due to caloric compensation, which ultimately caused reduced weight gain when compared to the treated groups. In our study, no caloric compensation occurred in the control groups as the rats were receiving normal drinking water, and thus no significant differences in food intake and weight gain

were observed (Figure 4.1), although the blood glucose level was relatively high in the saccharin consuming diabetic group (Figure 4.2).

Insulin resistance and β -cell dysfunction lead to chronic hyperglycemia, the clinical hallmark of T2D (Nyenwe et al., 2011). The type 2 diabetic rats in the present study had increased weekly blood glucose, reduced serum insulin and impaired oral glucose tolerance. Although treatment with saccharin did not alter these parameters; serum fructosamine level was significantly reduced in both normal and diabetic rats. Serum fructosamine reflects of average glucose concentration over 2-3 weeks (Shin et al., 2013). Since 3h-FBG and fasting blood glucose (before OGTT) were not affected, it is possible that saccharin reduces formation of serum fructosamine during the postprandial state which was not assessed in this study, and this would ultimately decrease the serum level of fructosamine in the treated rats. Further studies are required to confirm this contradictory finding. However, the non-significantly reduced serum insulin concentration and apparently reduced pancreatic islets sized in saccharin consuming normal and diabetic rats support the results of lower fructosamine concentrations in our study (Table 4.1). Additionally, serum fructosamine concentrations might also be increased due to the significant damage of kidney and pancreatic tissues (Figure 4.6).

Diabetic dyslipidemia is very common in patients with T2D, and studies have indicated that serum lipid abnormalities are associated with increased risks of cardiovascular events in both diabetic and non-diabetic individuals (Turner et al., 1998; ADA, 2004). Although the most common pattern of dyslipidemia in T2D is increased serum triglycerides and decreased HDL-cholesterol; in our study, T2D provoked increased triglycerides without affecting HDL-cholesterol. The results also indicate that saccharin treatment in both diabetic and normal rats influences the serum lipid profile of these rats. These findings are in good accordance with the study by Adbelaziz and Ashour (2011), which reported decreased serum triglycerides and total cholesterol in response to saccharin administration. It is possible that saccharin administration decreases serum lipids by acting directly or indirectly to lipid metabolism or lipid peroxidation (Abdelaziz and Ashour 2011). Interestingly, in the present study significant reduction in the serum lipids of the treated groups led to the improvement of the liver and

heart tissue morphology, particularly in the diabetic group. However, considering other data of this study, saccharin consumption may be beneficial for normal individual but may not be for people with T2D.

Although, abnormally elevated liver function enzymes (ALT and AST), ALP and LDH in the serum of type 2 diabetic patients have been reported (Celik et al., 2002; Harris, 2005), some reports including the present study have suggested that LDH is not always affected by diabetes (Oliver et al., 1993). Induction of these serum enzymes usually indicates tissue damage during diabetic condition (Shaheen et al., 2009). In our study, the elevated serum ALP and LDH concentrations in diabetic and normal rats, respectively, in response to saccharin administration could have resulted from changes in the kidney cellular morphology that was observed, ultimately causing leakage of these enzymes into circulation. In contrast to the current study, some reports have suggested that oral administration of saccharin can also elevate serum ALT, AST and ALP in normal rats and patients (Negro et al., 1994; Adbelaziz and Ashour, 2011; Andrejic et al., 2013). These abnormalities in serum enzymes may have been due to abnormally high doses of saccharin used in the previous studies, which led to changes in liver histology and ultimately leakage of these enzymes to the blood (Andrejic et al., 2013).

4.6 Conclusions

The results of the present study indicate that the consumption of commercially available saccharin may not have favourable effects with regards to serum lipid profile, organ histology and inflammatory biomarkers in both normal and diabetic rats. However, further clinical study is needed to confirm these effects in humans.

4.7 References

Please refer to the reference section at the end of this thesis (pages 126-144).

CHAPTER FIVE

Effects of commercially available aspartame-containing sweetener in an experimentally induced rat model of type 2 diabetes

Siphiwe Ndumiso Dlamini, Nondumiso Prosperity Mbambo, Md. Shahidul Islam*

Biomedical Research Lab, Department of Biochemistry, University of KwaZulu-Natal (Westville Campus), Durban 4000, South Africa

***Corresponding author:**

Dr MS Islam, Department of Biochemistry, School of Life Sciences, University of KwaZulu-Natal (Westville Campus), Durban 4000, South Africa

TEL: +27 31 260 8717; FAX: +27 31 260 7942; E-mail: islAMD@ukzn.ac.za

Running title:

Effects of aspartame on diabetes

5.1 Abstract

Non-nutritive sweeteners (NNS) such as aspartame have become highly popular as sugar substitutes for diabetic patients. Majority of studies have reported conflicting results and have used aspartame in its pure form which is not consumed by people. Hence, the present study primarily aimed at investigating the effects of commercially available aspartame-based sweetener on diabetes related parameters and its associated complications in both normal and type 2 diabetes rat model. Seven-week-old male Sprague-Dawley rats were randomly divided into four groups, namely: Normal Control (NC), Toxicological Aspartame (TASP), Diabetic Control (DBC) and Diabetic Aspartame (DASP). Type 2 diabetes was induced experimentally in the DBC and DASP groups only and rats with blood glucose concentrations >300 mg/dl were considered as diabetic. During the 13 week experimental period, the control groups received normal drinking water, whilst TASP and DASP groups were administered with aspartame-containing NNS solutions, *ad libitum*, at concentrations equivalent to the sweetness of 20% and 10% sucrose solution, respectively. Treatment with toxicological aspartame resulted in significantly reduced serum fructosamine and creatinine concentrations in normal rats, when brain, heart, liver, kidney and pancreatic tissue morphologies were significantly damaged in both normal and diabetic rats. The data of this study suggest that although aspartame-based NNS may not have higher degree of detrimental effects on diabetes associated parameters, the significantly damaged morphology of the major organ tissues as well related serum biomarkers both in normal and diabetic conditions raised a big concern of its use as a NNS and also in various food and food products.

5.2 Introduction

Diabetes Mellitus (DM) is a global health problem and affects more than 382 million people worldwide (IDF Atlas, 2013). This disease is classified into two major forms; type 1 and type 2, where type 2 accounts for 90-95% of all diabetes cases. According to Ripsin et al. (2009), patients with type-2 diabetes (T2D) should implement healthy lifestyle changes; such as reducing calorie intake in their diet and increasing physical activity to manage the disease. Moreover, high dietary intake of sucrose and fructose, especially in the form of liquid, has been consistently linked to increased energy intake and body weight gain, increased risk of developing T2D, decreased insulin sensitivity, lipid dysregulation, and visceral adiposity (Schulze et al., 2004; Dhingra et al., 2007; Stanhope et al., 2009; Hu and Malik, 2010). Thus, non-nutritive sweeteners (NNSs) have become highly popular as sugar substitutes for individuals with diabetes, since these substances have high sweetening power without any undesired calories. Several studies have been conducted to assess whether NNSs possess health risks as food additives or not (Benton, 2005; Whitehouse et al., 2008; Brown et al., 2010; Yang 2010; Brown and Rother, 2012) and reports of these studies have been largely conflicting , with conclusions ranging from safe at all concentrations to unsafe at any concentration.

Aspartame is a dipeptide NNS that is used in many food and drinks as a sugar substitute and has been subject to a number of controversies (Renwick and Nordmann, 2007; Tandel, 2011). Although this NNS has been approved in more than 90 countries, it has been banned in some countries due to concerns about long-term carcinogenic effects and side effects such as headaches that have been frequently reported in humans (Halldorsson et al., 2010). Studies that have been conducted recently on aspartame have largely focused on the effect on body weight gain (Feijo et al., 2013), hepatotoxicity (Abhilash et al., 2011), neurotoxicity (Christian et al., 2004; Bergstrom et al., 2007), and cancer (Soffritti et al., 2006; Gallus et al., 2007). However, the research on the effect of this NNS on diabetes-related parameters is very limited, although it is mainly being consumed by diabetic individuals. Moreover, the majority of studies have been carried out using aspartame in its pure form, which is not what people are consuming. Recently, it has been reported that aspartame may have an additive or synergistic effect when used in combination with other food additives (Lau et al., 2006;

Collison et al., 2012). Given that aspartame is usually used in combination with other NNSs to achieve the sweetness profile similar to sucrose, we hypothesized that the effect of aspartame in the form in which it is consumed by people has different effects to what has been previously reported.

Therefore, the primary objective of this study was to assess the effects of commercially available aspartame that is used as a sugar substitute, on diabetes related parameters and its associated complications. The study also aimed at investigating the toxicological effects of aspartame in normal rats.

5.3 Materials and methods

Please refer to chapter 2 of this thesis (pages 30-39) for details.

5.4 Results

5.4.1 Food and fluid intake, and body weight

Figure 5.1 shows the mean food and fluid intake and body weight gain for the 13 week experimental period. Although, aspartame did not affect daily food and fluid intake, administration of a high dose of this NNS for 13 week provoked a non-significantly ($p > 0.05$) increased weight gain in the normal rats, whilst the diabetic rats were not affected by the low dose of aspartame treatment (Figure 5.1).

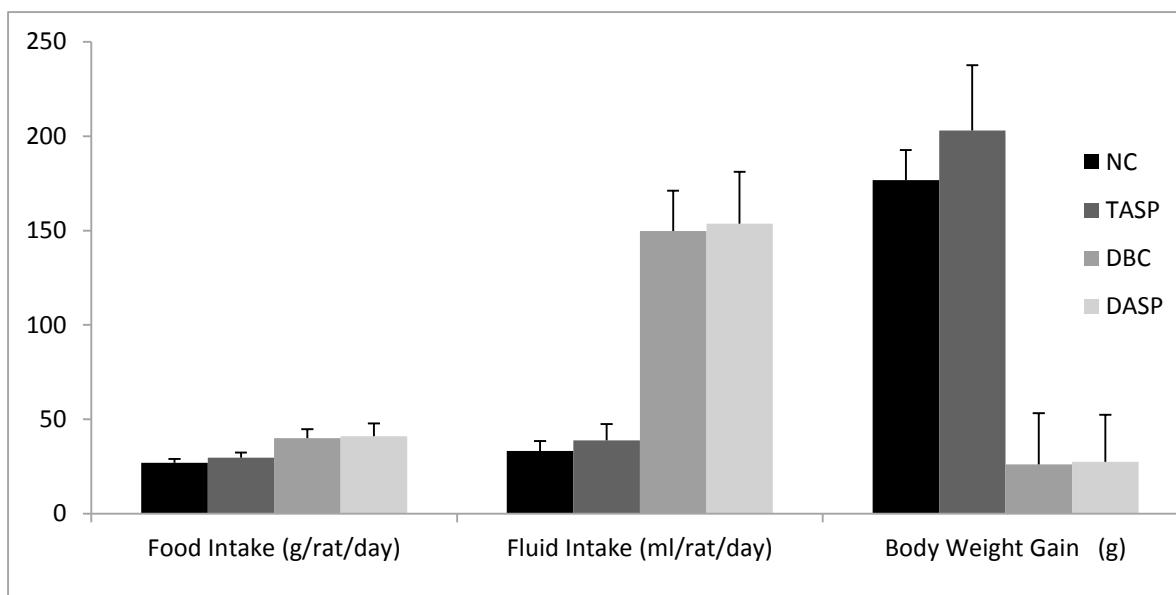


Figure 5.1: Mean food and fluid intake and body weight gain during the entire experimental period. Data presented as mean \pm SD of 5-6 rats. NC, Normal Control; TASP, Toxicological Aspartame; DBC, Diabetic Control; DASP, Diabetic Aspartame.

5.4.2 Weekly Blood Glucose and OGTT

Figure 5.2 shows weekly blood glucose concentrations of all groups over the 13 week experimental period. During the entire experimental period the diabetic groups had significantly elevated blood glucose compared to the normal groups. No significant differences were caused by treatment with the aspartame-containing NNS, in both diabetic and normal groups (Figure 5.2), which has been further confirmed by the calculated area under the curve (AUC) presented in Fig. 5.3.

The OGTT was performed at week 12 and data are shown in Fig. 5.4. The oral glucose tolerance was not significantly affected by aspartame administration, in both diabetic and normal rats as well (Figure 5.4).

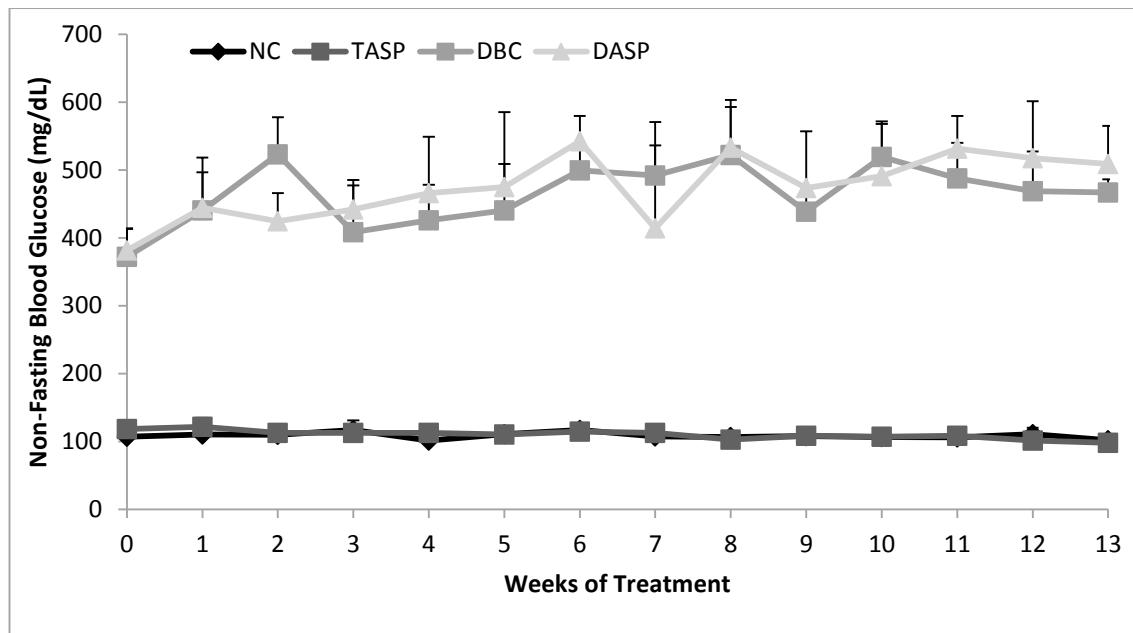


Figure 5.2: Mean weekly non-fasting blood glucose in different rats groups over the 13 week experimental period. Data are presented as mean \pm SD of 5-6 rats. NC, Normal Control; TASP, Toxicological Aspartame; DBC, Diabetic Control; DASP, Diabetic Aspartame.

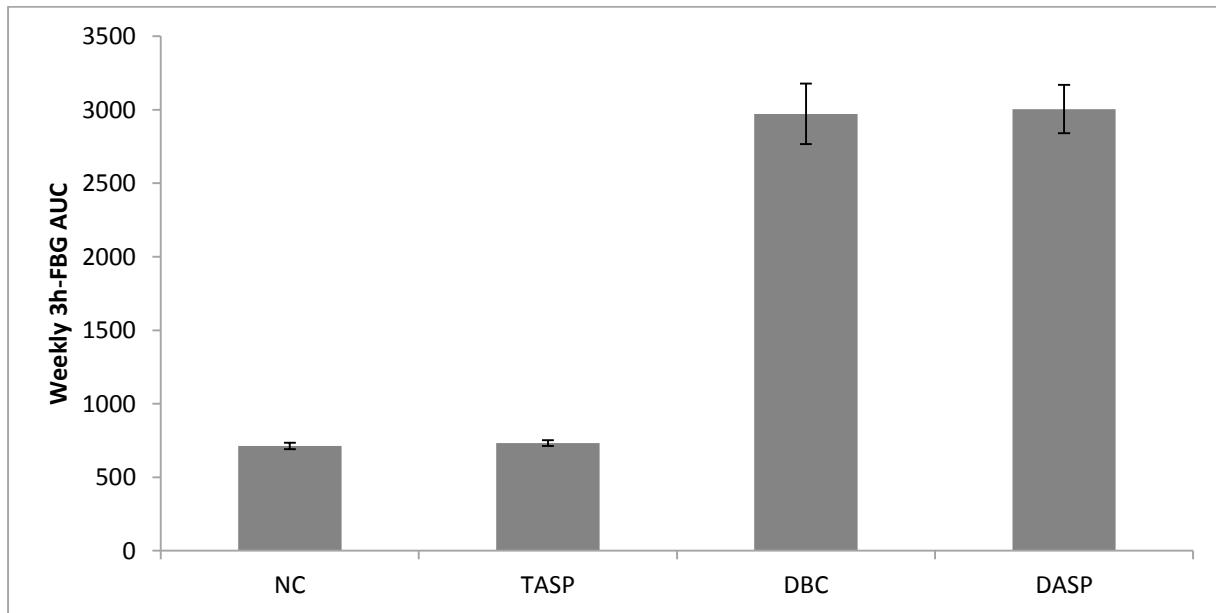


Figure 5.3: Calculated area under the curve (AUC) for weekly non-fasting blood glucose in different rats groups over the 13 week experimental period. Data are presented as mean \pm SD of 5-6 rats. NC, Normal Control; TASP, Toxicological Aspartame; DBC, Diabetic Control; DASP, Diabetic Aspartame.

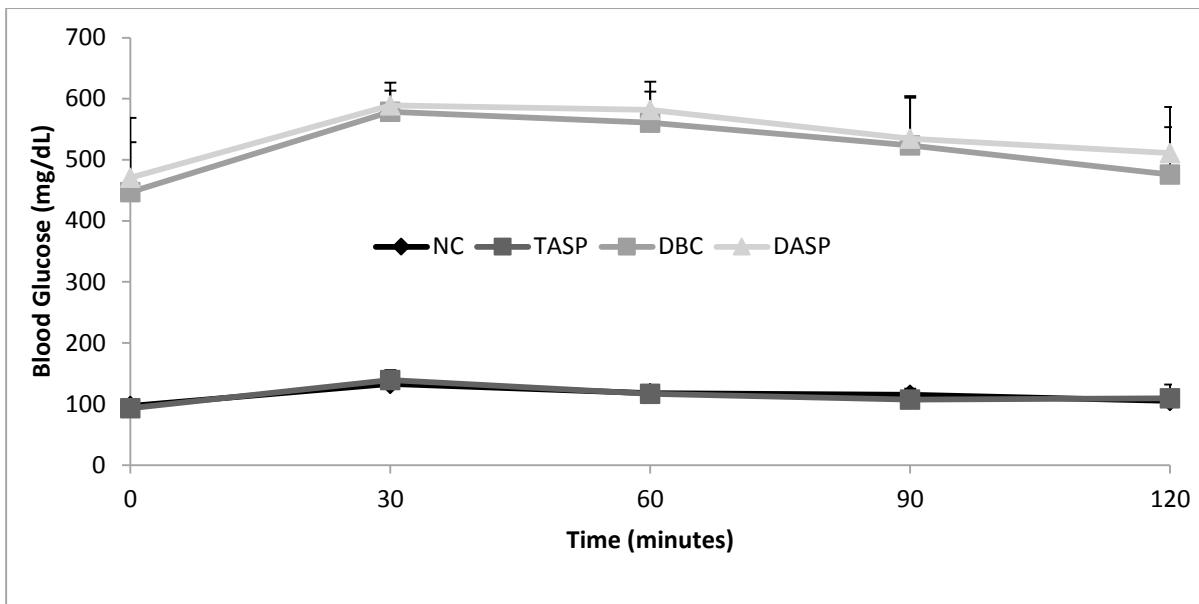


Figure 5.4: Oral glucose tolerance test (OGTT) in different animal groups at week 12 of the 13-week experimental period. Data are presented as mean \pm SD of 5-6 rats. NC, Normal Control; TASP, Toxicological Aspartame; DBC, Diabetic Control; DASP, Diabetic Aspartame.

5.4.3 Serum lipid profile

The serum lipid profile at 13 weeks is shown in Figure 5.5. There was no significant difference when comparing all groups for serum total cholesterol and HDL cholesterol. The diabetic control group only had significantly increased serum triglycerides, whilst both triglycerides and LDL cholesterol were significantly elevated in the diabetic rats treated with aspartame compared to their corresponding normal groups (Figure 5.5).

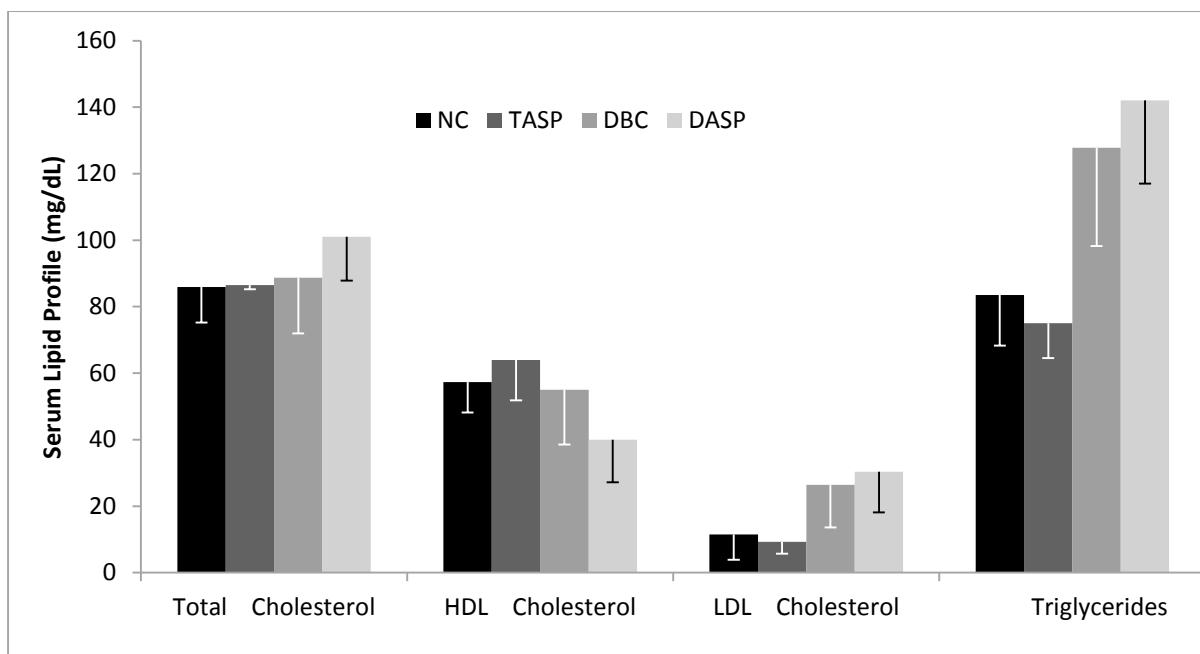


Figure 5.5: Serum lipid profile in the different animal groups at the end of the experimental period. Data are presented as mean \pm SD of 5-6 rats. NC, Normal Control; TASP, Toxicological Aspartame; DBC, Diabetic Control; DASP, Diabetic Aspartame.

5.4.4 Liver function enzymes in the serum

Figure 6 represents the concentrations of serum alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) of all groups at 13 weeks. The results indicated that administration of aspartame has no influence on these enzymes (Figure 5.6).

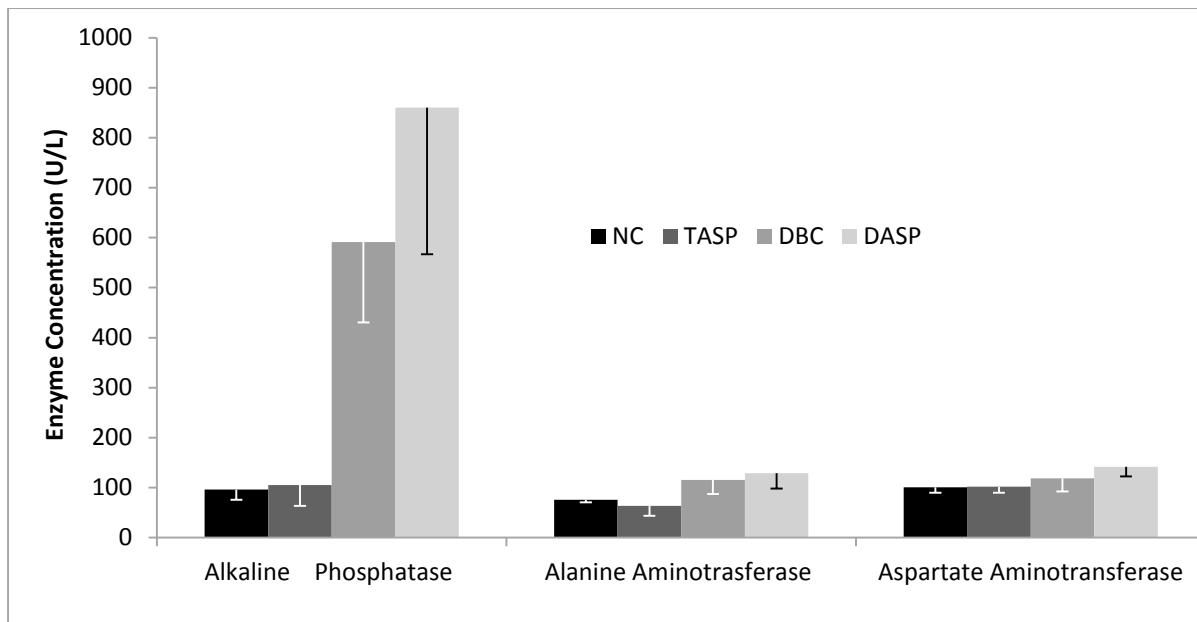


Figure 5.6: Concentrations of alkaline phosphates and liver function enzymes in the serum of different animal groups at the end of the experimental period. Data are presented as mean \pm SD of 5-6 rats. NC, Normal Control; TASp, Toxicological Aspartame; DBC, Diabetic Control; DASp, Diabetic Aspartame.

5.4.5 Serum insulin, fructosamine, LDH, CK-MB and creatinine

High dose of aspartame administration provoked a non-significant reduction in serum insulin but a significant decrease in serum fructosamine, whilst a low dose aspartame in the diabetic group did not cause any changes. Although diabetes did not significantly affect serum LDH and CK-MB, administration of a low dose aspartame provoked insignificant increases in these enzymes. Administration of a high dose aspartame to normal rats caused a significant reduction in the serum creatinine, without affecting serum uric acid when these parameters were not affected by the supplementation of lower dose of aspartame to diabetic rats (Table 5.1).

Table 5.1: Serum insulin, fructosamine, LDH, CK-MB, uric acid, and creatinine concentrations at the end of the 13-week experimental period

	NC	TASP	DBC	DASP
Insulin (ng/L)	152.51 ± 56.18	104.44 ± 33.77	74.74 ± 8.08	72.15 ± 3.44
Fructosamine (μmol/L)	505.40 ± 31.39	428.80 ± 30.9 [*]	588.80 ± 30.34	582.00 ± 48.25
LDH (U/L)	1129.00 ± 295.00	1070.00 ± 142.41	2270.5 ± 1183.71	3434.4 ± 524.87
CK-MB (U/L)	1331.98 ± 669.40	1112.26 ± 323.79	1926.52 ± 587.81	2618.98 ± 646.11
Uric acid (mg/dL)	9.44 ± 3.46	11.88 ± 2.48	9.74 ± 0.98	10.84 ± 2.67
Creatinine (mg/dL)	4.73 ± 0.87	2.34 ± 0.53 [*]	3.89 ± 0.87	4.66 ± 0.59

Data presented as mean ± SD of 5-6 rats. * Significantly different compared to NC group, (Paired *t*-test, *p* < 0.05). NC, Normal Control; TSCL, Toxicological Sucralose; DBC, Diabetic Control; DASP, Diabetic Sucralose; LDH, Lactate dehydrogenase; CK-MB, Creatine kinase (heart specific).

5.4.6 Histopathological findings

Administration of an aspartame-containing NNS to both diabetic and non-diabetic rats for 13 week, led to histological changes in the liver, heart, kidney and pancreatic tissues (Figure 5.7,. 5-20) along with the brain of diabetic rats (Figure 5.7, 3 & 4). The histopathological findings show that administration of aspartame led to enhanced tissue damage to the liver (Figure 5.7, 5-8) and heart (Figure 5.7, 9-12) organs, in both diabetic and normal rats. Moreover, aspartame also provoked increased vacuolation in the renal tubules (yellow arrows) and size of the Bowmans space (BS) in the kidneys (Figure 5.7.13-16) of both normal and diabetic rats. In addition, aspartame treatment resulted in reduced size of the islets of Langerhans (IL) in the non-diabetic rats, and improvements in the histology of the pancreatic acini (A) in both diabetic and normal rats (Figure 17-20).

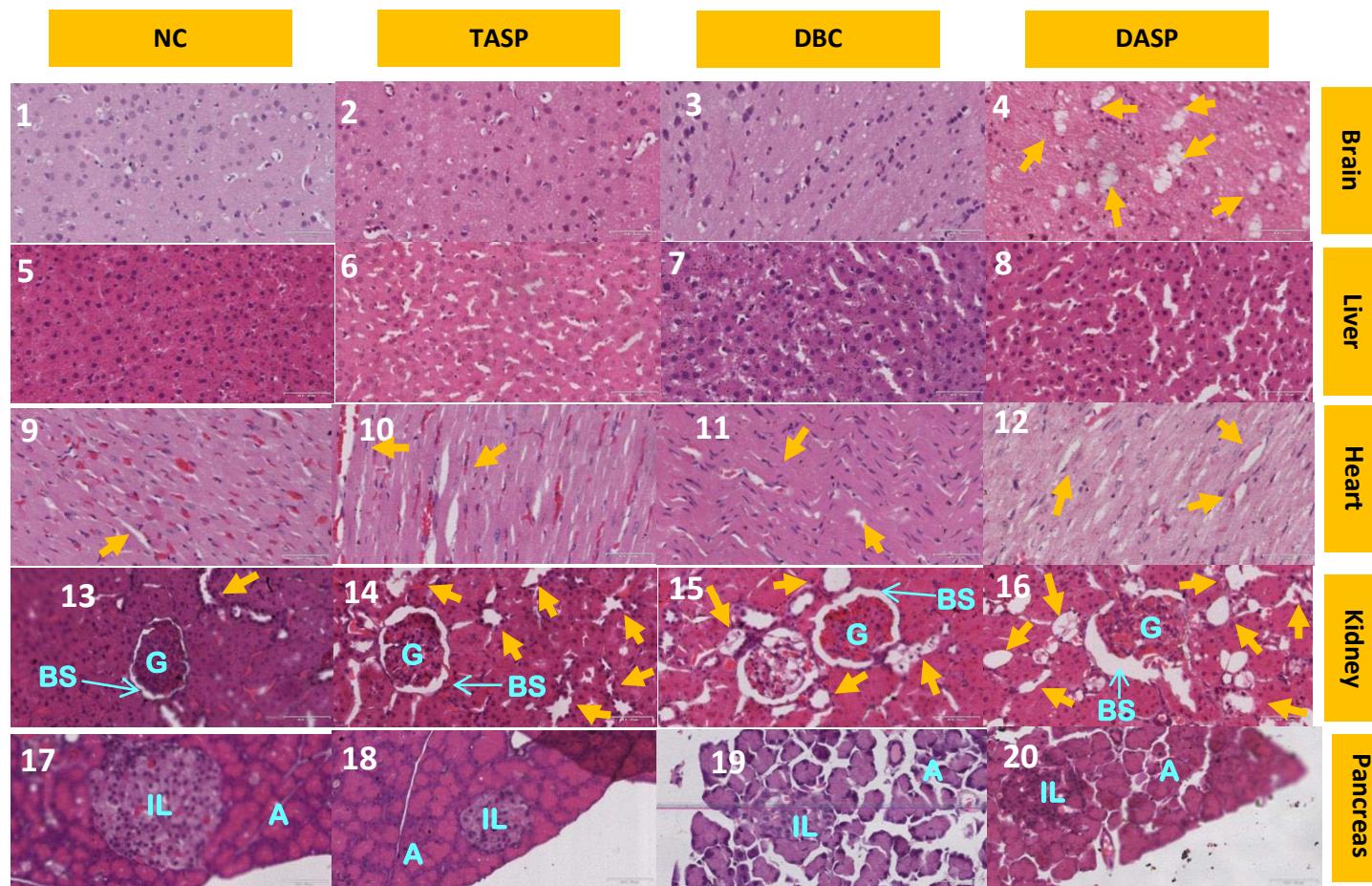


Figure 5.7: Histological examinations of the brain (1-4), liver (5-8), heart (9-12), kidney (13-16) and pancreas (17-20) of different animal groups at the end of the experimental period, at X400 total magnification. NC, Normal Control; TASP, Toxicological Aspartame; DBC, Diabetic Control; DASP, Diabetic Aspartame; BS, Bowmans Space; G, Glomerulus; IL, Islet of Langerhans; A, Acini.

5.5 Discussion

Although aspartame is one of the most widely used NNSs particularly by diabetic and obese individuals, use of this NNS has not been approved in some countries due concerns about long-term carcinogenic effects and frequently reported side effects (Halldorsson et al., 2010). Thus, its safety for consumption by humans remains highly debatable. Additionally, studies that have been conducted to date have used aspartame in its pure form, and not in the form in which it is consumed by people. Hence this study primarily aimed at assessing the effects of aspartame in the form in which it is available commercially, in normal and T2D rat model. The key findings of the study was that consumption of aspartame may not have significant effects on major diabetes related parameters but causes severe organ damage both in normal and type 2 diabetic rat model. .

The most common symptoms of untreated diabetes include polyuria, polydipsia, polyphagia, unexplained weight loss, and sometimes impaired vision (ADA, 2007). Some studies have suggested that aspartame consumption can lead to increased food intake and body weight gain in animal models (Kim et al., 2011; Collison et al., 2012^b; Feijo et al., 2013). In this study, aspartame consumption did not significantly affect food and fluid intake, and body weight gain (Figure 5.1), after 13 weeks of treatment. Nonetheless, the non-significantly elevated body weight gain, in normal rats treated with high dose aspartame, might suggest that treatment with aspartame over longer periods or using a higher dose could have resulted to a statistically significant increase in body weight. It is important to note that food intake was not affected by aspartame treatment and thus could not be related to the non-significantly elevated weight gain observed (Figure 5.1). Feijó et al. (2013) also reported an increased body weight gain in rats receiving yogurt sweetened with aspartame for 12 weeks compared to rats receiving sucrose sweetened yogurt. Both groups were receiving a similar caloric intake and it was speculated that weight gain may have resulted from a decrease in energy expenditure. Moreover, a recent study revealed that, administration of an aspartame-containing sweetener to obese mice causes a decrease in oxygen consumption, which ultimately leads to increased adiposity after 4 weeks of

administration (Mitsutomi et al., 2014). Hence, aspartame-based NNS may have body weight gaining effects which supports our findings.

Diabetes is a disease characterized by chronic hyperglycemia, which leads to changes in metabolites of lipids, carbohydrates and blood coagulation factors, and ultimately resulting in complications such as diabetic cardiomyopathy, nephropathy, neuropathy and retinopathy. It is well established that glycemic control plays a major role in the prevention of these complications. Thus a number of diabetic patients substitute sucrose with NNSs such as aspartame in order to achieve better glycemic control. Although acute and short term studies have reported that aspartame does not affect glucose homeostasis (Anton et al., 2010), some long term studies have demonstrated that aspartame exposure may promote increased blood glucose and insulin intolerance (Collison et al., 2012^a; Collison et al., 2012^b). In our study, the results indicate that consumption of the commercially available aspartame-based sweetener for 13 weeks did not alter non-fasting blood glucose (Figure 5.2), in normal and diabetic rats. This was also confirmed by calculated area under the curve (Figure 5.3) and supported by the results of oral glucose tolerance test (Figure 5.4) which showed no effect of aspartame treatment on glucose tolerance. The results were also corroborated with serum insulin which was decreased as a result of type-2 diabetes and not aspartame treatment, in the diabetic rats (Table 5.1). Although, these results indicate that aspartame does not influence blood glucose concentrations, the reduced serum fructosamine and non-significantly reduced serum insulin show that a high dose of aspartame in normal rats reduces these parameters in the blood.

According to the American Diabetes Association (2004), diabetic dyslipidemia is very common in individuals with T2D. Decreased HDL-cholesterol level and elevated triglycerides concentration in the serum is the most common dyslipidemia pattern in patients with T2D. Additionally, increased LDL-cholesterol concentrations may also be present in this form of diabetes (ADA, 2004). In this study, non-significantly increased total cholesterol, LDL-cholesterol and triglyceride in the aspartame consuming diabetic group compared to DBC group confirmed the induction of diabetic more dyslipidemia in this group (Figure 5.5). Relatively more elevated serum LDL-cholesterol due to aspartame treatment in the diabetic may have been caused by increased lipolysis of very low density

lipoproteins (VLDL), which serve as LDL precursors, by the lipoprotein lipase enzyme. Hence, long-term consumption of aspartame-based NNS may worsen dyslipidemia particularly in diabetic condition along with other organ specific complications.

Tissue damage is strongly associated with diabetes and increases in serum enzymes such as lactate dehydrogenase (LDH), alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) have been reported in diabetic condition (Celik et al., 2002; Harris, 2005; Shaheen et al., 2009). Elevated serum ALP level is usually used as a marker for tissue damage (Shaheen et al., 2009). Additionally, chronic elevation of these enzymes is common in T2D (Harris, 2005; Shaheen et al., 2009). In this study, although not-significantly but markedly elevated serum ALP in the diabetic rats treated with aspartame (Figure 5.6) was indicative of tissue damage, as confirmed by the histopathological examination (Figure 5.7). The results indicate that consumption of aspartame causes major organ tissue damage which worsens in diabetic condition. Abhilash et al. (2011) reported that chronic consumption of aspartame caused hepatic damage, when administered at 1000 mg/kg BW for 180 days in non-diabetic male Wistar rats. In their study, liver damage was also indicated by increased liver function enzymes and this was corroborated with the histopathological findings as well, which revealed leukocyte infiltration in rats treated with aspartame. The results of our study indicate that the increased serum LDH and CK-MB enzymes in the diabetic rats receiving aspartame due to enhanced organ tissue damage (Table 5.1). Since aspartame is rapidly metabolized into phenylalanine, aspartate and methanol (Christian et al., 2004); it is possible that the tissue damage observed in all organs examined could have resulted from increases in serum methanol. Methanol has been previously reported to cause changes in the surface charge density as well as oxidative stress, leading to leakage of some enzymes (Parthasarathy et al., 2006). Finally, lower creatinine level in the blood has been proposed as a risk factor for insulin resistance and T2D (Harita et al., 2009). In our study, significantly reduced creatinine concentrations were seen in the TASP group (Table 5.1), indicating that excess consumption of aspartame might increase the risk of developing insulin resistance and T2D.

5.6 Conclusions

In conclusion, the data of this study suggest that although aspartame-based NNS may not adversely affect diabetogenic parameters, the damaged morphology of metabolic organs and altered serum biomarkers both in normal and diabetic rats suggests caution for the use of aspartame in food products.

5.7 References

Please refer to the reference section at the end of this thesis (pages 126-144).

CHAPTER SIX

Effects of commercially available cyclamate containing sweetener in an experimentally induced rat model of type 2 diabetes

Siphiwe Ndumiso Dlamini, Nondumiso Prosperity Mbambo, Md. Shahidul Islam*

Biomedical Research Lab, Department of Biochemistry, University of KwaZulu-Natal (Westville Campus), Durban 4000, South Africa

***Corresponding author:**

Dr MS Islam, Department of Biochemistry, School of Life Sciences, University of KwaZulu-Natal (Westville Campus), Durban 4000, South Africa
TEL: +27 31 260 8717; FAX: +27 31 260 7942; E-mail: islAMD@ukzn.ac.za

Running title:

Effects of cyclamate on diabetes

6.1 Abstract

Studies on the effects of cyclamate have largely focused on the potential toxic effects on bladder and testicular cancers, and have used in its pure form. The present study aimed at evaluating the biological and toxicological effects of the commercially available cyclamate in diabetic and normal rats, respectively. Seven-week-old male Sprague-Dawley rats were randomly divided into four groups, as follows: Normal Control (NC), Toxicological Cyclamate (TCLM), Diabetic Control (DBC) and Diabetic Cyclamate (DCLM). Type 2 diabetes was induced experimentally in the DBC and DCLM groups only and rats with non-fasting blood glucose concentrations >300 mg/dl were considered as diabetic. During the 13 week intervention period, the control groups (NC and DBC) were provided with normal drinking water, whilst TCLM and DCLM groups received cyclamate-containing NNS solutions, *ad libitum*, at concentrations equivalent to 20% and 10% sucrose solution in terms of sweetness, respectively. Treatment with a toxicological dose of cyclamate in normal rats influenced the serum lipid profile, significantly ($p < 0.05$) reduced serum ALT, fructosamine, insulin and sizes of the pancreatic islets, and non-significantly reduced serum AST, ALP, LDH and CK-MB. Conversely, treatment with a low dose cyclamate in diabetic rats, led to significantly ($p < 0.05$) increased serum ALT and non-significantly elevated AST, ALP, LDH and CK-MB in the serum. Additionally treatment commercially available cyclamate also caused significant changes in the histology of the examined organs in both normal and diabetic rats. The results of the present study demonstrate that uncontrolled consumption of commercially available cyclamate can profoundly influence diabetes related parameters and its related complications. Individuals consuming this sweetener are advised to limit their daily intake.

6.2 Introduction

Excessive intake of sugar in the diet has been consistently associated with a number of detrimental health conditions. Some studies have reported that consumption of sugar decreased insulin sensitivity, lipid dysregulation and increased food intake, visceral adiposity, and risk of developing T2D (Dhingra et al., 2007; Malik et al., 2010; Schulze et al., 2004; Stanhope et al., 2009). Thus, there has been an

upsurge for the use of alternative sweeteners as sugar substitutes. Although a great number of sweeteners exist, non-nutritive sweeteners (NNSs) have gained more popularity because they are many times sweeter than sucrose and are not associated with undesired calories. According to Sylvetsky et al. (2012), in the United States intake of NNSs had increased from 6.1% to 12.5% among children and from 18.7% to 24.1% among adults, from 1999 to 2008.

Moreover, since NNSs are recommended for use by diabetic patients, a number of diabetic patients are substituting sugar with these types of sweeteners (Gougeon et al., 2004). The principle behind the use of NNSs by diabetic individuals is that the patients can enjoy a number of sweetened foods and drinks without negative impact on blood glucose control.

The high use of NNSs brings the issue of safety especially with regards to uncontrolled daily consumption. The earliest NNS-related safety issue started in 1969 when a study reported that high doses of cyclamate when administered in a 10:1 ratio with saccharin increased the incidence of bladder cancer in male rats (Price et al., 1970). Subsequently, cyclamate was banned by the United States Food and Drug Administration (USFDA) in 1970, which is still banned in the USA and other parts of the world till today. Nonetheless, since no studies have reported an association between cyclamate and cancer in humans, this NNS has been approved by the European Scientific Committee on Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at an acceptable daily intake of 11 mg/kg, and is used as a food additive in more than 50 countries at the moment (Yang, 2010). Studies on the effects of cyclamate have largely focused on the toxicity in testes and urinary bladders (Creasy et al., 1990; Takayama et al., 2000).

However, since cyclamate is also consumed in sugar-free drinks and products, and sugar substitute by diabetic patients, investigations on the effects on glucose homeostasis and other diabetes related parameters are necessary. Moreover, majority of the studies have used cyclamate in its pure form and not in the form that is consumed by people. Additionally, recent studies have reported that NNS may have synergistic or additive effects when used with other food additives (Collison et al., 2012; Lau et al., 2006). Since cyclamate is usually used in combination with other NNSs such as acesulfame potassium (Ace-K), and contains other ingredients such as maltodextrin and anticaking agents; we

hypothesized that the commercially available form of cyclamate may have different effects to what have been previously reported. Therefore, in the present study, the biological and toxicological effects of commercially available cyclamate are investigated in an experimentally induced rat model of type 2 diabetes and in normal rats.

6.3 Materials and methods

Please refer to chapter 2 of this thesis (pages 30-39) for details.

6.4 Results

6.4.1 Daily food and fluid intake, and body weight gain

Figure 6.1 presents the data for daily food and fluid intake, and body weight gain at the end of the experimental period. Although, diabetes induction provoked the induction of daily food and fluid intake, and reduction of body weight gain; treatment with cyclamate did not result in any significant differences in these parameters. Nonetheless, cyclamate markedly increased fluid intake and body weight gain both in normal and diabetic rats (Figure 6.1).

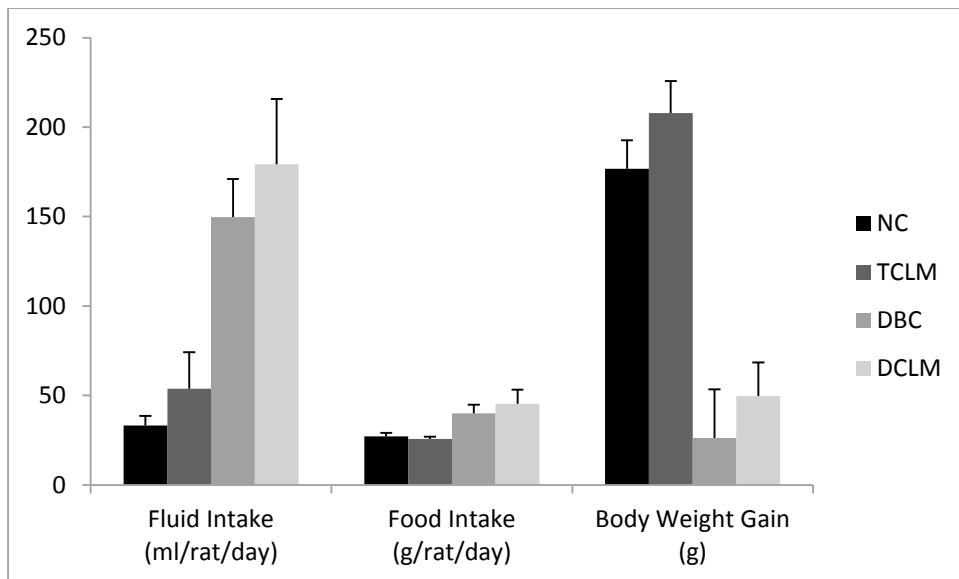


Figure 6.1: Mean food and fluid intake and body weight gain during the entire experimental period. Data presented as mean \pm SD of 5-7 rats. NC, Normal Control; TCLM, Toxicological Cyclamate; DBC, Diabetic Control; DCLM, Diabetic Cyclamate.

6.4.2 Weekly blood glucose and oral glucose tolerance test

The data for weekly blood glucose over the 13 week experimental period are presented in Figure 6.2. The graph shows that diabetes caused an elevation in blood glucose concentrations throughout the entire study period, when compared to the non-diabetic groups (NC and TCLM). Cyclamate administration did not affect the blood glucose concentration almost entire experimental period except week 6. Administration of a high dose of cyclamate to the normal rats also did not have any significant effect on blood glucose concentration when compared to the respective normal control group (Figure 6.2).

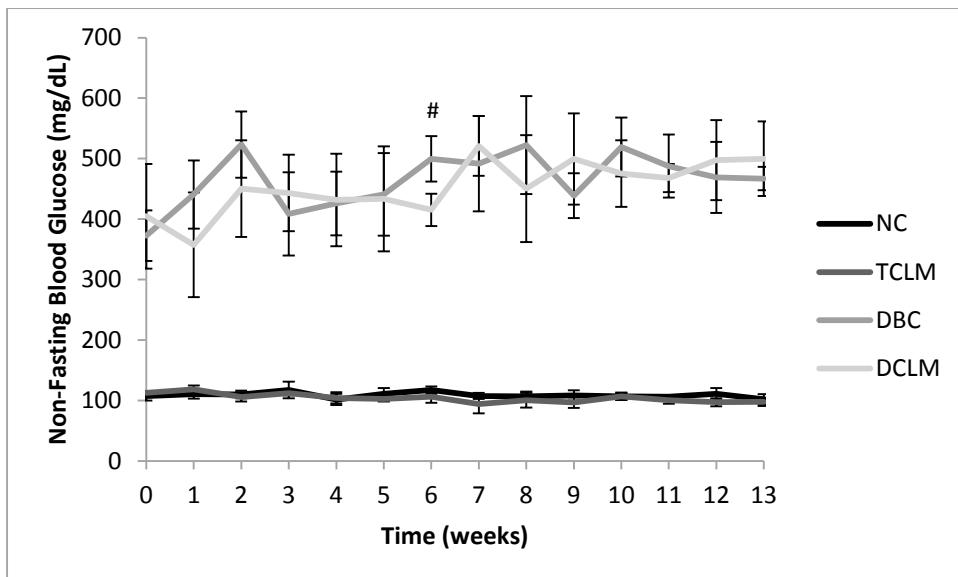


Figure 6.2: Weekly non-fasting blood glucose in different rat groups over the 13 week experimental period. Data are presented as mean \pm SD of 5-6 rats. [#] Significantly different compared to NC and DBC groups, respectively (Paired t-test, $p < 0.05$). NC, Normal Control; TCLM, Toxicological Cyclamate; DBC, Diabetic Control; DCLM, Diabetic Cyclamate.

The OGTT was done at the 12th week of the intervention period and Figure 6.3 shows the data from this test. Although, blood glucose was increased in the diabetic groups (DBC and DCLM) in comparison to the non-diabetic groups; treatment with cyclamate had no significant effects on the blood glucose concentrations during the entire period of the OGTT (Figure 6.3).

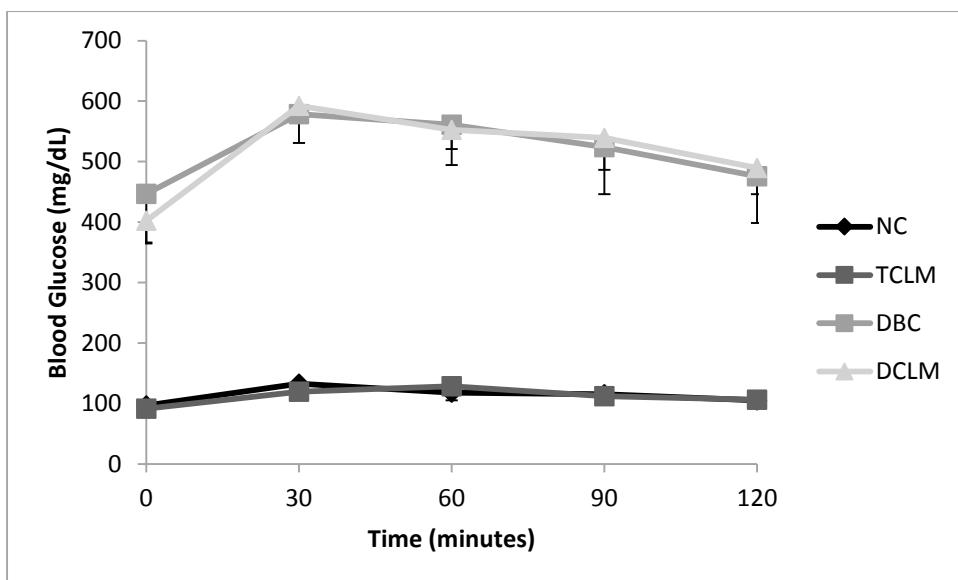


Figure 6.3: Oral glucose tolerance test (OGTT) in different animal groups at week 12 of the 13 week experimental period. Data are presented as mean \pm SD of 5-7 rats. NC, Normal Control; TCLM, Toxicological Cyclamate; DBC, Diabetic Control; DCLM, Diabetic Cyclamate.

6.4.3 Serum lipid profile and serum enzymes

Data for lipid profile and enzymes in the serum are shown in Figure 6.4. Although a low dose of commercially available cyclamate had no effect on serum lipid profile in the diabetic rats, a high dose of this sweetener led to significantly ($p<0.05$) increased serum LDL-cholesterol and significantly ($p<0.05$) reduced serum triglyceride concentrations, in the normal rats. Total cholesterol and HDL-cholesterol were not influenced by cyclamate consumption (Fig. 6.4).

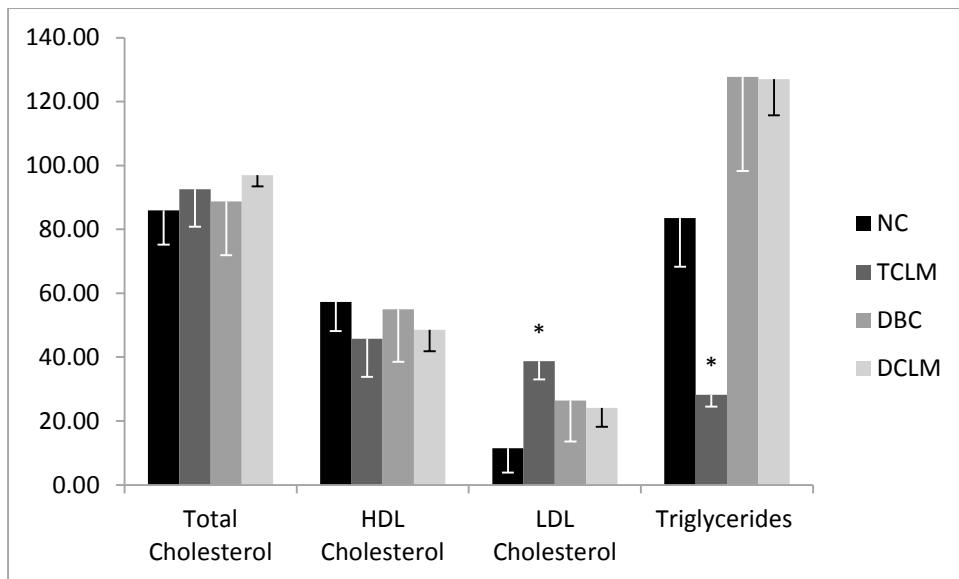


Figure 6.4: Serum lipid profile in the different animal groups at the end of the experimental period. Data are presented as mean \pm SD of 5-6 rats. *Significantly different from normal control group (Paired t- test, $p < 0.05$). NC, Normal Control; TCLM, Toxicological Cyclamate; DBC, Diabetic Control; DCLM, Diabetic Cyclamate; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein.

Interestingly, treatment with cyclamate for 13 week significantly ($p < 0.05$) reduced ALT in normal rats when significantly ($p < 0.05$) elevated in diabetic rats compared to their respective controls Table 6.1. Serum AST, ALP, LDH and CK-MB concentrations were not significantly affected by the consumption of cyclamate either in normal or in diabetic rats Table 6.1.

Table 6.1: Serum lipid profile and serum enzymes at the end of the experimental period

Serum Enzymes	NC	TCLM	DBC	DCLM
ALT	75.80 ± 4.97	$62.60 \pm 5.77^*$	115.60 ± 28.40	$151.14 \pm 20.61^\#$
AST	101.00 ± 10.93	89.40 ± 14.54	118.80 ± 26.41	133.40 ± 22.77
ALP	96.40 ± 20.84	81.40 ± 14.66	591.00 ± 160.53	851.80 ± 256.86
LDH	1129.00 ± 295.00	723.40 ± 211.23	2270.50 ± 1183.71	3184.20 ± 387.21
CK-MB	1331.98 ± 669.40	614.00 ± 108.33	1926.52 ± 587.81	2144.94 ± 767.45

Data presented as mean \pm SD of 5-7 rats. * and # significantly different compared to NC and DBC groups, respectively (Paired t-test, $p < 0.05$). NC, Normal Control; TCLM, Toxicological Cyclamate; DBC, Diabetic Control; DCLM, Diabetic Cyclamate; ALT, Alanine Transaminase; AST, Aspartate Transaminase; ALP, Alkaline Transaminase; LDH, Lactate Dehydrogenase; CK-MB, Creatine Kinase (Heart Specific).

6.4.4 Serum insulin, fructosamine, uric acid and creatinine concentrations

Consumption of a high dose commercially available cyclamate in normal rats resulted in significantly ($p<0.05$) reduced serum insulin and fructosamine, with no significant effects on serum uric acid and creatinine. Conversely, treatment with a low dose of cyclamate in diabetic rats had no influence on these parameters (Figure 6.5).

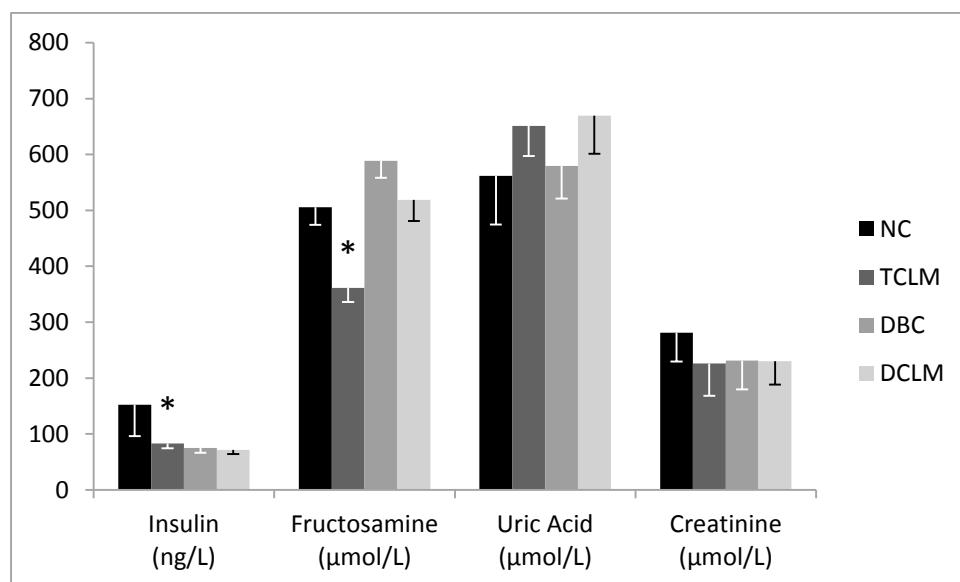


Figure 6.5: Serum insulin, fructosamine, uric acid and creatinine concentrations of different animal groups at the end of the experimental period. Data expressed as mean \pm SD of 5-7 rats. *

Significantly different compared to NC and DBC groups, respectively (Paired t-test, $p < 0.05$). NC, Normal Control; TCLM, Toxicological Cyclamate; DBC, Diabetic Control; DCLM, Diabetic Cyclamate.

6.4.5 Histopathological findings

The results from histopathological studies are shown in Figure 6.6. Examination of the liver tissue (Figure 6.6.5-8) revealed that cyclamate consumption induced hepatic tissue damage in both diabetic and non-diabetic rats, when compared to their respective controls. In addition, cyclamate increased tissue damage in the kidney (Figure 6.6.13-16), as marked by increased sizes of the Bowman's spaces

(BS) and vacuolation in the convoluted tubules (Shown with yellow arrows). Regarding the pancreatic tissue, cyclamate led to noticeably reduced sizes of the Islets of Langerhans (IL) in normal rats only, and significantly reduced cellularity in the IL in both normal and diabetic rats (Figure 6.6.17-20). Supplementation of cyclamate had no effect on the cellular morphology of the brain tissue (Figure 6.6.1-4) in this experiment.

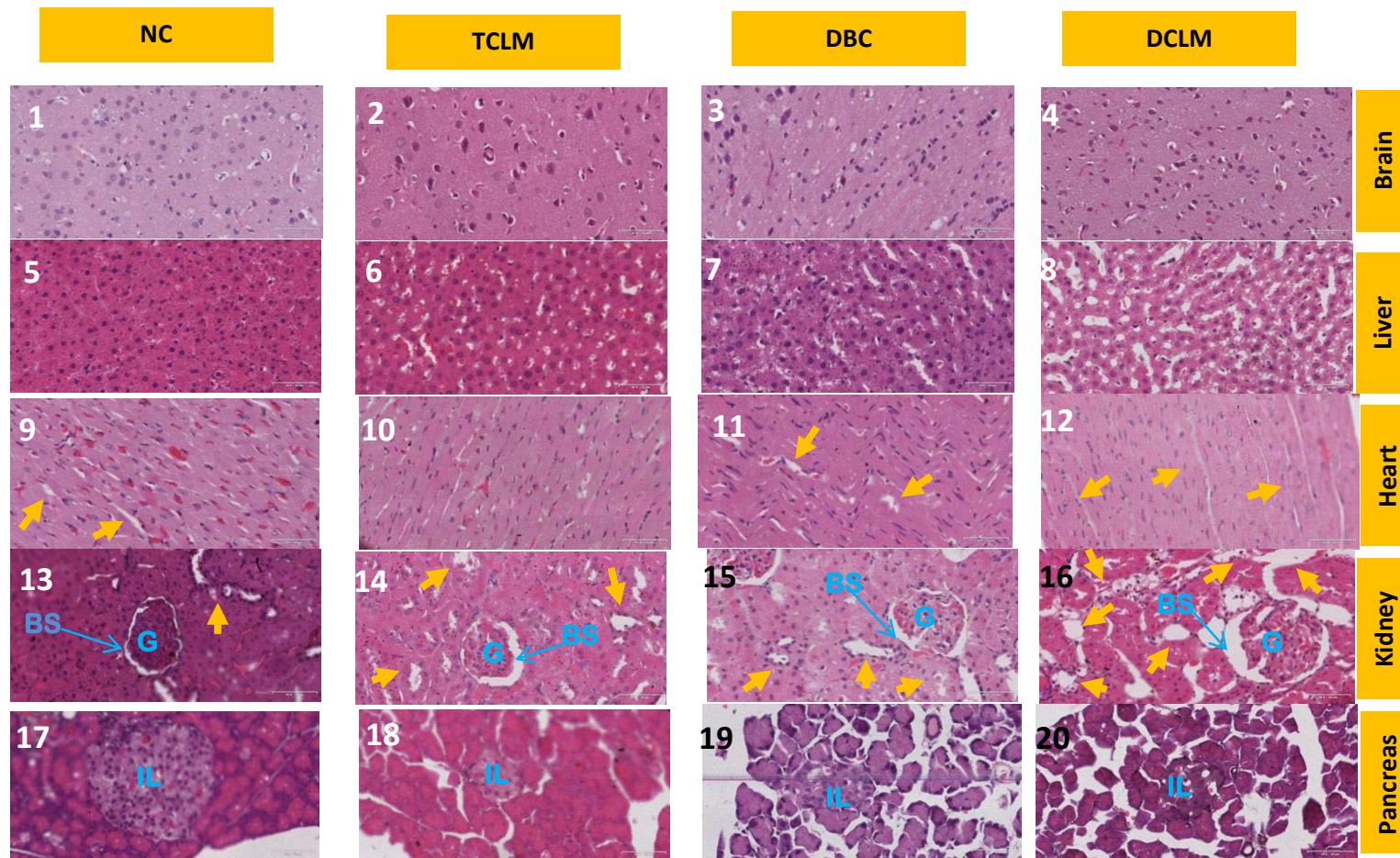


Figure 6.6: Histological examinations of the brain (1-4), liver (5-8), heart (9-12), kidney (13-16) and pancreas (17-20) of different animal groups at the end of the experimental period; at 400x total magnification. NC, Normal Control; TCLM, Toxicological Cyclamate; DBC, Diabetic Control; DCLM, Diabetic Cyclamate.

6.5 Discussion

Although several studies have assessed the safety of cyclamate, majority of the studies have used cyclamate in its pure form. The main aim of the current study was to investigate the biological effects of commercially available cyclamate in an experimentally induced rat model of T2D. The study also assessed the toxicological effects of this sweetener at a higher dose in normal rats.

Diabetes is generally characterized by chronic hyperglycemia, which leads to polydipsia, polyphagia and unexplained weight loss, some of the most common symptoms of this disease (ADA, 2013). In our study the diabetic rats exhibited chronic hyperglycemia during the entire experimental period (Figure 6.2), which resulted in increased food and fluid intake, as well as reduced body weight gain (Figure 6.1). Additionally, T2D is a form of diabetes that is characterized by progressive decline in insulin action (insulin resistance) and inability of β -cells to compensate for insulin resistance (β -cell dysfunction) (Nyenwe et al., 2007). In this study, impaired glucose homeostasis, as marked by glucose intolerance (Figure 6.3) and significantly reduced serum insulin concentrations (Figure 6.4) in the diabetic groups, were indicative of the major characteristics of type 2 diabetes.

Although no significant differences were observed with regards to polydipsia, polyphagia and body weight among the normal and diabetic rats; the non-significant elevation of fluid and food intake, and body weight gain due to cyclamate supplementation suggest that longer intervention period could have significantly increased these parameters. Previous studies have shown that bacterial flora of the gastrointestinal tract in rats can convert cyclamate into cyclohexylamine, a metabolite that was associated with changes in water intake and body weight in monkeys when administered at 34 mg/kg for 4 weeks (Takayama et al., 2000). Thus it is possible that the non-significantly elevated fluid intake and weight gain in rats treated with cyclamate was due to the presence of cyclohexylamine, although further studies are required for confirmation.

Interestingly, consumption of cyclamate had no influence on weekly blood glucose, but significantly and non-significantly reduced serum fructosamine in normal and diabetic rats, at a toxicological and low dose, respectively. According to Shin et al. (2013), serum fructosamine can be used to determine

average blood glucose concentrations over 2-3 weeks. Since in this study only 3h-FBG and overnight fasting blood glucose (during OGTT) were assessed, it is possible that cyclamate may reduce blood glucose at a different state such as postprandial. Reduction of blood glucose during the postprandial state could have ultimately reduced the amount of fructosamine production in the blood and the concentration of insulin produced by the pancreas since a similar pattern was observed with regards to serum insulin concentration.

It is well documented that type 2 diabetes can lead to a number of complications and these include dyslipidemia and organ tissue damage which leads to leakage of enzymes into circulation. Thus diabetic patients are encouraged to eat healthy and reduce calorie intake in their diets in order to avoid these complications (Ripsin et al., 2009). In the present study, the type 2 diabetes led to dyslipidemia which was marked by significantly increased triglycerides and non-significantly elevated LDL-cholesterol in the serum (Figure 6.4). Significantly reduced serum triglycerides and significantly increased serum LDL-cholesterol in the normal rats only (treated with a toxicological dose) indicated that cyclamate administration for 13 week either influences serum lipid profile only in normal rats or only at a higher dose. Although a dose dependent study is required to confirm this hypothesis, but it is possible that cyclamate elevated LDL-cholesterol by increasing the synthesis of very low density lipoproteins, which act as precursors for LDL-cholesterol.

Although several animal and human studies have suggested that cyclamate has no toxic effect (Renwick et al., 2004; Takayama et al., 2000), some reports including the current study have indicated detrimental effects of this sweetener at cellular or tissue level (Martins et al., 2005; Sasaki et al., 2002). In the present study administration of the commercially available cyclamate for 13 week caused noticeable changes in the histology of the examined tissues (Figure 6.6), and this ultimately influenced the enzyme concentrations in the serum (Table 6.1). The tissue damage may have been due to the conversion of cyclamate into cyclohexylamine, a toxic metabolite that has been extensively studied in animals and humans (Buss et al., 1992; Creasy et al., 1990; Renwick et al., 2004). In a previous study, Martins et al. (2005) observed the cellular alterations in fetal liver of rats after intraperitoneal administration of cyclamate (60 mg/kg bw) during pregnancy. In our study, the

damage of the liver tissue in cyclamate consuming groups is in accordance with the results obtained by Martins et al. (2005). Additionally, Creasy et al. (1990) reported that high doses of cyclohexylamine can cause vacuolation in some sensitive tissues in male rats. The vacuolations in the renal tissue of cyclamate consuming groups are also in line with the above-mentioned study.

6.6 Conclusion

The present study demonstrated that commercially available cyclamate containing sweetener influences some of the diabetes-related parameters in both normal and diabetic rats, at toxicological and low dose, respectively. Uncontrolled consumption of this non-nutritive sweetener appears to induce and worsen organ tissue injury in both normal and diabetic rats, respectively, and ultimately influence serum enzyme concentration. Thus, individuals using cyclamate are advised to limit their daily intake.

6.7 References

Please refer to the reference section at the end of this thesis (pages 126-144).

CHAPTER SEVEN

Commercially available stevia-based sweetener, anti-diabetic or toxic: An *ad libitum* feeding study in a type 2 diabetes model of rats

Siphiwe Ndumiso Dlamini, Nondumiso Prosperity Mbambo, Md. Shahidul Islam*

Biomedical Research Lab, Department of Biochemistry, University of KwaZulu-Natal (Westville Campus), Durban 4000, South Africa

***Corresponding author:**

Dr MS Islam, Department of Biochemistry, School of Life Sciences, University of KwaZulu-Natal (Westville Campus), Durban 4000, South Africa
TEL: +27 31 260 8717; FAX: +27 31 260 7942; E-mail: islammd@ukzn.ac.za

Running title:

Effects of stevia on diabetes

7.1 Abstract

The use of non-nutritive sweeteners as sugar substitutes by overweight, obese and diabetic individuals has been increased rapidly in recent years, when Stevia is the most popular one not only due to its natural source of origin but also for its anti-diabetic potential. However, the effects commercially available stevia-based NNS are not investigated at all. The present study was investigated the effects of the *ad libitum* consumption of commercially available stevia-based NNS in an experimentally-induced rat model of type 2 diabetes (T2D). Seven-week-old male Sprague-Dawley rats were randomly divided into three groups, namely: Normal Control (NC), Diabetic Control (DBC) and Stevia (STV). T2D was induced in the DBC and STV groups only. The control groups were received normal drinking water, whilst rats in the STV group were administered with stevia-containing NNS solution, at a concentration equivalent to the sweetness of 10% sucrose. After 13 week intervention, although food and fluid intake, body weight, weekly blood glucose, glucose tolerance, serum insulin, fructosamine, ALT, ALP, creatinine and brain, liver and pancreatic histopathology were not significantly affected; serum AST, LDH and CK-MB as well as heart and kidney histopathology were significantly deteriorated in stevia-based NNS consuming group compared to the DBC group. The data of this study suggest that uncontrolled consumption of stevia-based NNS has no significant anti-diabetic effect but have some toxicological effects in an animal model of T2D. Hence, diabetic patients consuming stevia-based NNS should limit their daily intake.

7.2 Introduction

Excessive dietary intake of high-calorie sweeteners, such as sucrose and fructose, has been consistently associated with a number of detrimental health outcomes, such as elevated food intake, body weight gain and visceral adiposity, reduced insulin sensitivity, lipid dysregulation and risk of developing type 2 diabetes (T2D) (Schulze et al., 2004; Dhingra et al., 2007; Stanhope et al., 2009; Malik et al., 2010). Therefore, there has been an upsurge on the use of alternative sweetening agents, such as non-nutritive sweeteners (NNSs), as sugar substitutes. NNSs have gained great popularity,

especially to overweight, obese and diabetic individuals, due to their extremely higher sweetness levels with no undesired calories compared to sucrose. The most widely used NNSs are aspartame, saccharin, acesulfame-potassium (Ace-K), sodium cyclamate, sucralose, neotame and stevia.

Majority of these NNSs are chemically synthesized and several studies have reported conflicting results in terms of their safety for human consumption. Although previous clinical studies have suggested that NNSs have no adverse biological effects when consumed at concentrations below the acceptable daily intake (ADI), the effects of the chronic consumption of these NNS on food intake, body weight and adiposity, impaired glucose homeostasis, bladder cancer, and liver toxicity are still conflicting (Whitehouse et al., 2008; Yang, 2010). However, according to data from number of recent studies, the natural originated NNSs are becoming more popular to consumers not only due to their sweetening power but also for their beneficial effects on health.

Stevia is a naturally-occurring NNS that has been reported to have a number of health benefits in both humans and experimental animals (Gregersen et al. 2004; Saravanan et al. 2012) when most of these studies were investigated its anti-diabetic potentials. Anti-oxidative, anti-diabetic and renal protective effects of stevia leaves and its various extracts and extracted polyphenols and fibers have been reported in streptozotocin- and alloxan-induced diabetic rats (Shivanna et al. 2013; Kujur et al. 2010). Rebaudioside A, a major constituent of Stevia rebaudiana, has been reported to have beneficial effects on glucose homeostasis in STZ-induced diabetic rats (Saravanan et al. 2012) and stimulated insulin secretion in isolated mouse islets (Abudula et al. 2004), however it did not improve glycemic control or blood pressure in spontaneously diabetic Goto-Kakizaki rats (Dyrskog et al. 2005). Although a number of previous studies assessed the effects of stevia on metabolic parameters in diabetic animals, the animals were not consuming commercially available stevia containing sweetener (Gregersen et al. 2004; Chang et al. 2005).

On the other hand, recent animal studies have indicated that some food additives may have synergistic or additive effects when used in combination (Lau et al., 2006; Collison et al., 2012). According to the Canderel website (2014), commercially available stevia tablet is composed of lactose, steviol glycosides, favourings, emulsifier (croscarmellose sodium), anticaking agents (calcium salts of fatty

acids, silicon dioxide). Thus, due to these constituents, stevia in the form in which it is consumed by people is composed of approximately 68% carbohydrates and 3% fat on weight to weight bases, and has about an energy value of 1286 KJ per 100 g (Canderel website, 2014). According to Savita et al. (2004), stevia in its pure form is composed of 52% carbohydrates, 3% fat, and has an energy value of 270 KJ.

Thus due to these differences, we hypothesized that stevia in the commercially available form would have different effects to what has been previously reported. As per our knowledge, no study on the detailed effects of stevia or its glycosides has been conducted on different organ histopathology and organ specific blood parameters until today. Therefore, the aim of the present study was to assess the effects of long-term uncontrolled consumption of commercially available stevia-based NNS in an experimentally induced rat model of T2D.

7.3 Materials and methods

Please refer to chapter 2 of this thesis (pages 30-39) for details.

7.4 Results

7.4.1 Calculation of the human equivalent dose of stevia glycosides

According to the results shown in Table 7.1, the concentration and corresponding consumption of stevia by the treated animals were 0.0462% and 75.06 mg per rat per day. The FDA recommends acceptable daily intake (ADI) of steviol is 4 mg/kg BW/ day which is equivalent to 12 mg/kg BW stevia glycosides (Beverage Institute for Health and Wellness), and the calculated human equivalent dose (ADI) for rats was determined to be 74.07 mg/kg BW, whilst the calculated actual consumption was 261.12 mg/kg BW.

Table 7.1: Calculation of the human equivalent dosages and actual consumption of stevia by the rats in drinking water

Mean fluid intake (ml/rat/day)	Mean body weight (g)	Concentration of stevia glycosides (%)	Consumption of stevia (mg/rat/day)	Human equivalent acceptable daily intake (ADI) (mg/kg/BW)	Actual Consumption (mg/kg BW)
162.47	287.45	0.0462	75.06	74.07	261.12

Data are presented as mean of 5-6 rats.

Human dose (mg/kg BW) = animal dose (mg/kg BW) x (animal K_m/ human K_m), where translated acceptable daily intake (ADI) of stevia glycosides for human is 12 mg/kg BW (The Beverage Institute for Health and Wellness) and K_m is 37 and rat K_m is 6. K_m is a correction factor which represents the relation between body weight and body surface area (Natural Research Institute).

Actual consumption (mg/kg BW) = [Consumption of sucralose (ml/rat/day) x 1000]/ Mean body weight (g)

7.4.2 Food and fluid intake, and weekly body weight change

The data for mean daily food and fluid intake over the 13 week experimental period are shown in Figure 7.1. The results showed that both food and fluid intake were significantly increased by the induction of diabetes and the consumption of stevia-based NNS did not significantly affect these parameters. Figure 7.2 shows the weekly body weight change over the 13 week experimental period. During the first three weeks (-3 to -1), there was no significant difference in body weight among all groups. After STZ injection, the body weight of diabetic groups (DBC and STV) was significantly reduced compared to the normal control group, which continued throughout the entire experimental

period. Although there was no significant difference between the body weight of DBC and STV group, the body weight of the STV-treated group was relatively better compared to the DBC group for the entire intervention period.

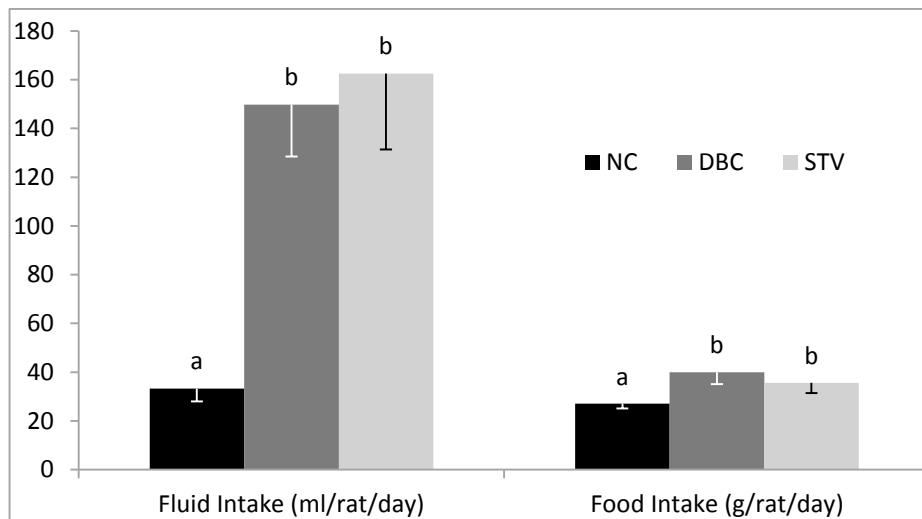


Figure 7.1: Mean food and fluid intake over the 13 week experimental period. Data presented as mean \pm SD of 5-6 rats. ^{a,b}Different letters over the bars for a given parameter are significantly different from each other groups of rats (Tukey's HSD multiple range *post-hoc* test, $p < 0.05$). NC, Normal Control; DBC, Diabetic Control; STV, Stevia.

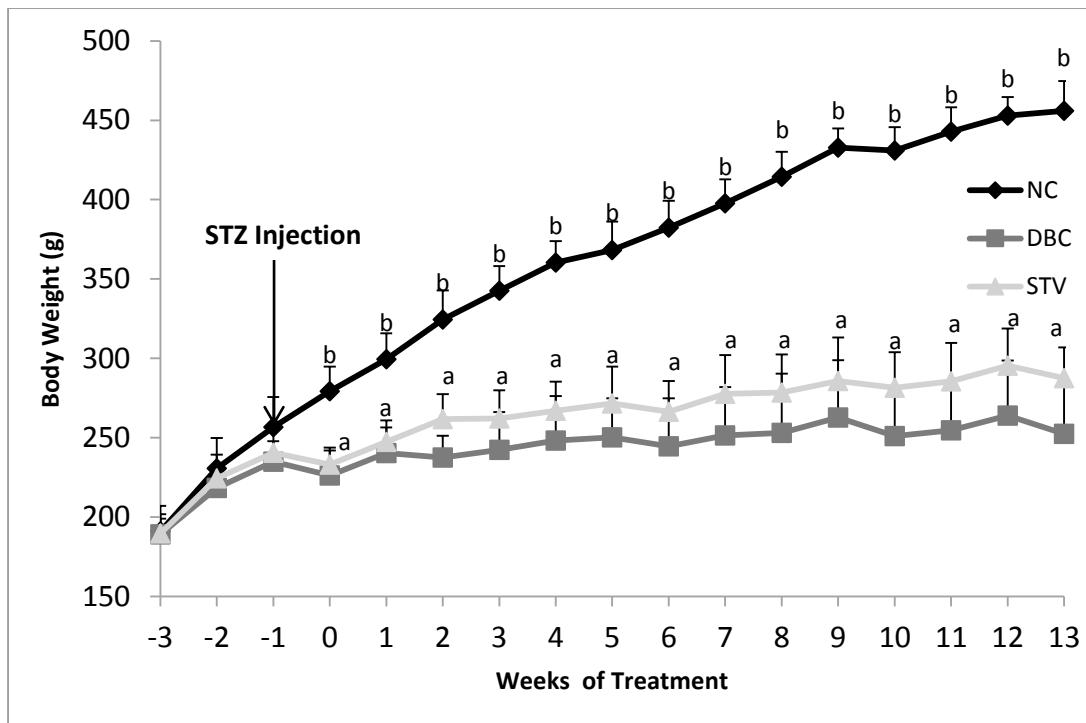


Figure 7.2: Mean weekly body weight during the entire experimental period. Data expressed as mean \pm SD of 5-6 rats. ^{a,b}Different letters over the lines for a given week are significantly different from each other groups of rats (Tukey's HSD multiple range *post-hoc* test, $p < 0.05$). NC, Normal Control; DBC, Diabetic Control; STV, Stevia.

7.4.3 Weekly non-fasting blood glucose and oral glucose tolerant test

Figure 7.3 represents the results of weekly blood glucose for the entire 13 week experimental period. Although there was no significant difference of blood glucose between the DBC and STV group in most of the weeks, the blood glucose of STV group was consistently lower in the treated group for the entire intervention period.

The oral glucose tolerance test (OGTT) was performed at week 12 of the 13-week experimental period and the results of this test are shown in Figure 7.4. Blood glucose concentrations were significantly increased in the diabetic groups compared to the normal control, during the entire experimental period. Treatment with stevia did not significantly influence the glucose tolerance level during the entire test period.

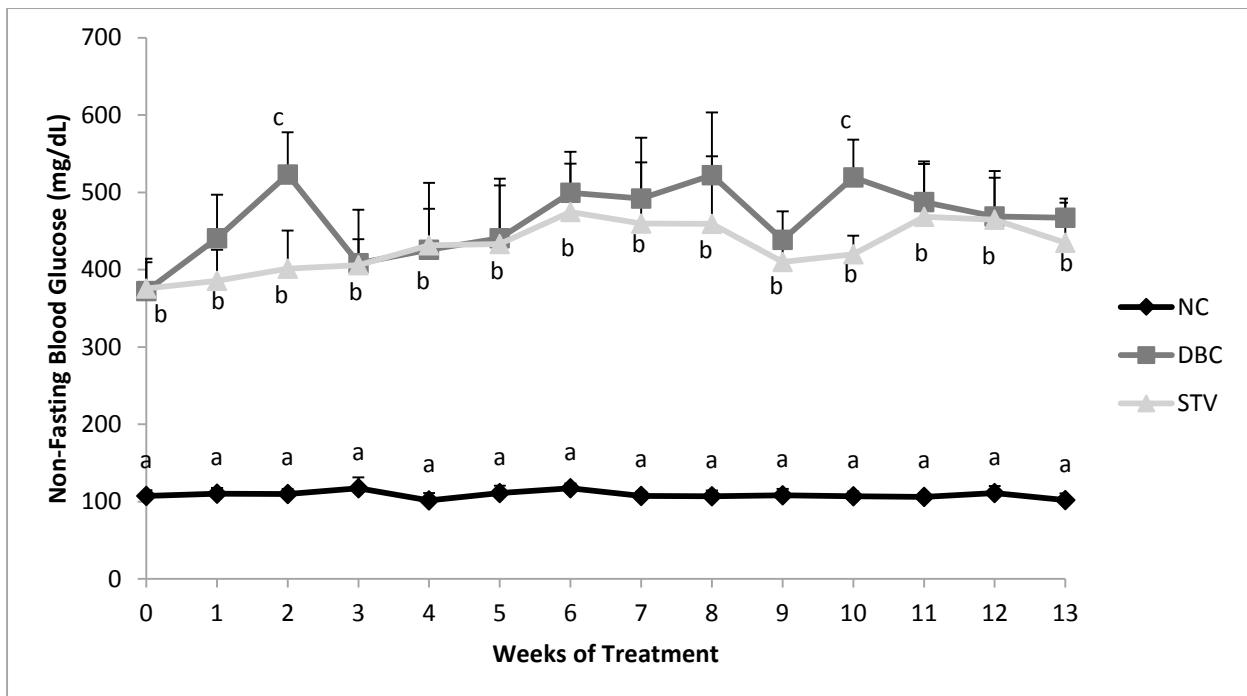


Figure 7.3: Mean weekly non-fasting blood glucose in different rat groups over the 13 week experimental period. Data are expressed as mean \pm SD of 5-6 rats. ^{abc}Different letters presented above the lines for a given week are significantly different from each other group of rats (Tukey's HSD multiple range *post-hoc* test, $p < 0.05$). NC, Normal Control; DBC, Diabetic Control; STV, Stevia.

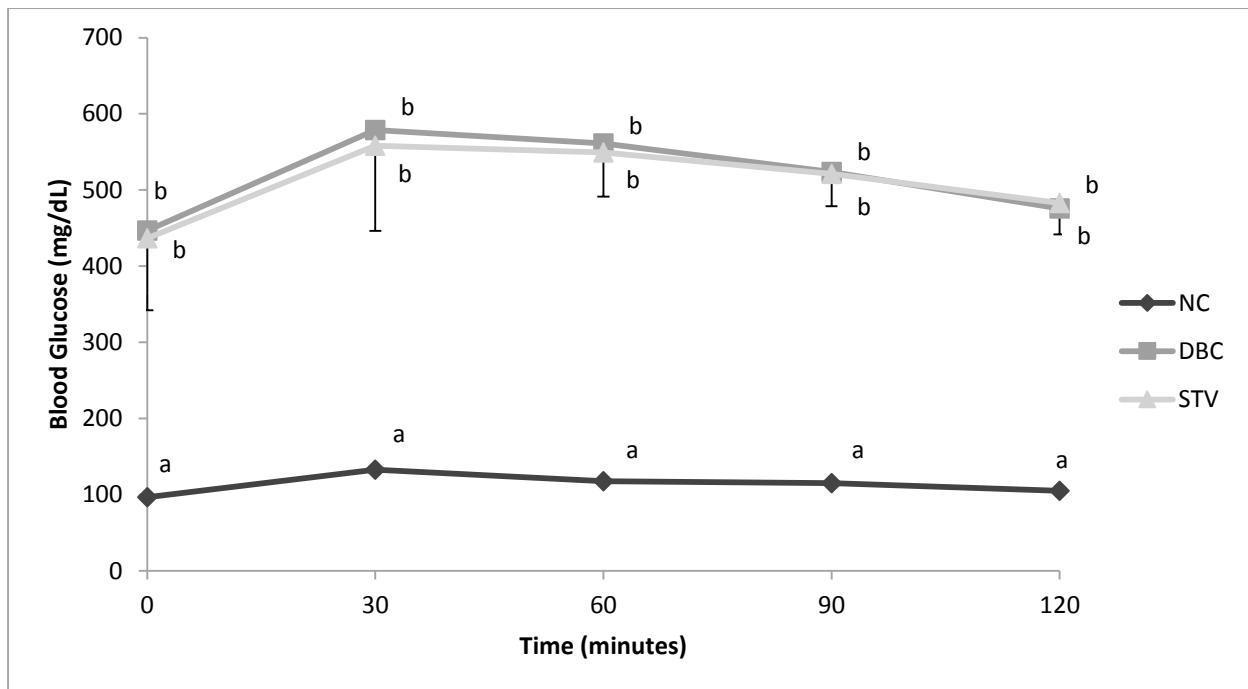


Figure 7.4: Oral glucose tolerance test (OGTT) in different animal groups at week 12 of the 13 week experimental period. Data are presented as mean \pm SD of 5-6 rats. ^{ab}Different letters presented above the lines for a given time are significantly different from each other group of rats (Tukey's HSD multiple range *post-hoc* test, $p < 0.05$). NC, Normal Control; DBC, Diabetic Control; STV, Stevia.

7.4.4 Serum insulin, fructosamine, uric acid and creatinine concentrations

Figure 7.5 represents the concentrations of serum insulin, fructosamine, uric acid and creatinine concentrations in the different animal groups at the end of the experimental period. Diabetes caused a significant decrease in insulin concentrations and a significant increase in fructosamine in the serum, without significant changes in serum uric acid and creatinine concentrations. Supplementation of stevia-based NNS did not significantly influence any of these serum parameters except serum uric acid was markedly higher in the stevia-consumed group compared to the DBC group (Fig. 7.5).

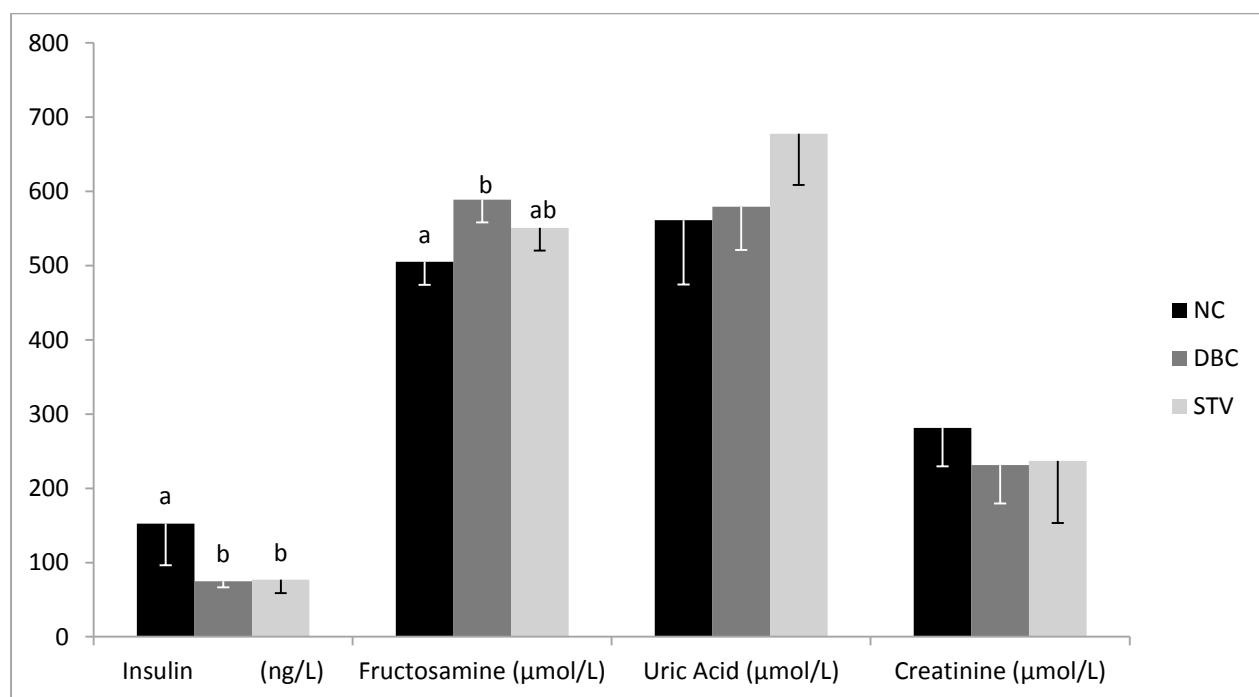


Figure 7.5: Serum insulin, fructosamine, uric acid and creatinine concentrations of different animal groups at the end of the experimental period. Data expressed as mean \pm SD of 5-6 rats.

^{ab}Different letters presented above the bars for a given time are significantly different from each other group of rats (Tukey's HSD multiple range *post-hoc* test, $p < 0.05$). NC, Normal Control; DBC, Diabetic Control; STV, Stevia.

7.4.5 Serum lipid profile and serum enzyme concentrations

Although induction of diabetes provoked a significant increase in the level of serum triglycerides, supplementation of a stevia-based NNS markedly reduced it almost to a normal level (Fig. 7.6). Other lipids were not affected by the treatment of stevia-based NNS.

The concentrations of serum enzymes at the end of the experimental period are shown in Table 7.2. Diabetes caused significant increases in serum ALT and ALP without affecting the other enzymes. On the contrary, treatment with stevia markedly but not significantly ameliorated these parameters when serum AST, LDH and CK-MB concentrations were significantly increased in the stevia consuming group compared to the DBC group (Table 7.2).

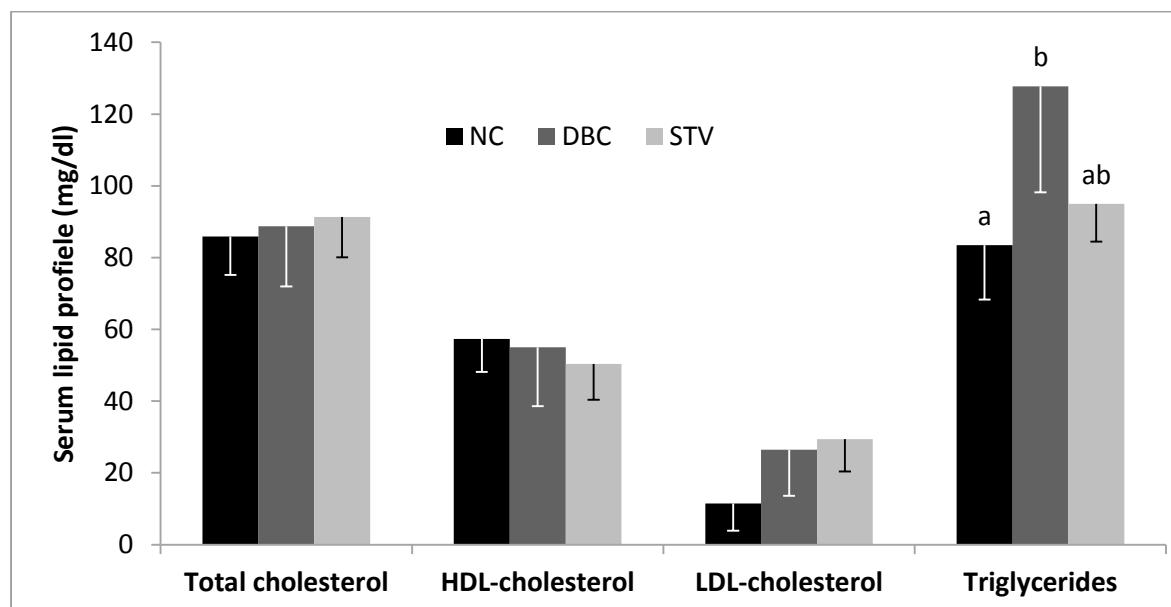


Figure 7.6: Serum lipid profile in different animal groups at the end of the 13 week experimental period. Data are presented as mean \pm SD of 5-6 rats. ^{ab}Different letters presented above the bars for a given parameter are significantly different from each other group of rats (Tukey's HSD multiple range *post-hoc* test, $p < 0.05$). NC, Normal Control; DBC, Diabetic Control; STV, Stevia.

Table 7.2: Serum enzymes at the end of the experimental period

Serum enzymes	NC	DBC	STV
Concentrations (U/L)			
ALT	75.80 ± 4.97^a	115.60 ± 28.40^b	94.60 ± 14.69^b
AST	101.00 ± 10.93^a	118.80 ± 26.41^a	136.50 ± 18.59^b
ALP	96.40 ± 20.84^a	591.00 ± 160.53^b	432.50 ± 123.83^b
LDH	1129.00 ± 295.00^a	2270.50 ± 1183.71^a	4702.33 ± 773.76^b
CK-MB	1331.98 ± 669.40^a	1926.52 ± 587.81^a	3424.80 ± 905.54^b

Data presented as mean \pm SD of 5-6 rats. ^{a,b}Different letters presented above the lines for a given time are significantly different from each other group of rats (Tukey's HSD multiple range *post-hoc* test, $p < 0.05$). NC, Normal Control; DBC, Diabetic Control; STV, Stevia; ALT, Alanine Transaminase; AST, Aspartate Transaminase; ALP, Alkaline Transaminase; LDH, Lactate Dehydrogenase; CK-MB, Creatine Kinase (Heart Specific).

7.4.6 Histopathological examination of organ tissues

The histopathological findings of the study are shown in Figure 7.7. Induction of diabetes led to noticeable changes in the histology of the liver, heart, kidney and pancreas (Figure 7.7.4-14). Diabetes did not seem to affect the cellular morphology of the brain tissue in the present study (Figure 7.7.1-2). In comparison to the normal control, severe damage to the liver and heart tissue, vacuolation and increased size of Bowmans space (BS) and convoluted tubules in the kidneys, and reduced size of the islets of Langerhans (IL) and damage to the acini in the pancreas was observed in the DBC group (Figure 7.7.4-14). On the other hand, supplementation of stevia significantly ameliorated damage to the liver (Figure 7.7.4-6) and acini of the pancreas (Figure 7.7.13-15), and reduced the BS in the kidney to its normal size (Figure 7.7.10-12). However, stevia also led to more severe cellular damage to the heart tissue (Figure 7.7.7-9), and vacuolation and more increased size of the convoluted tubules in the kidney (Figure 7.7.10-12).

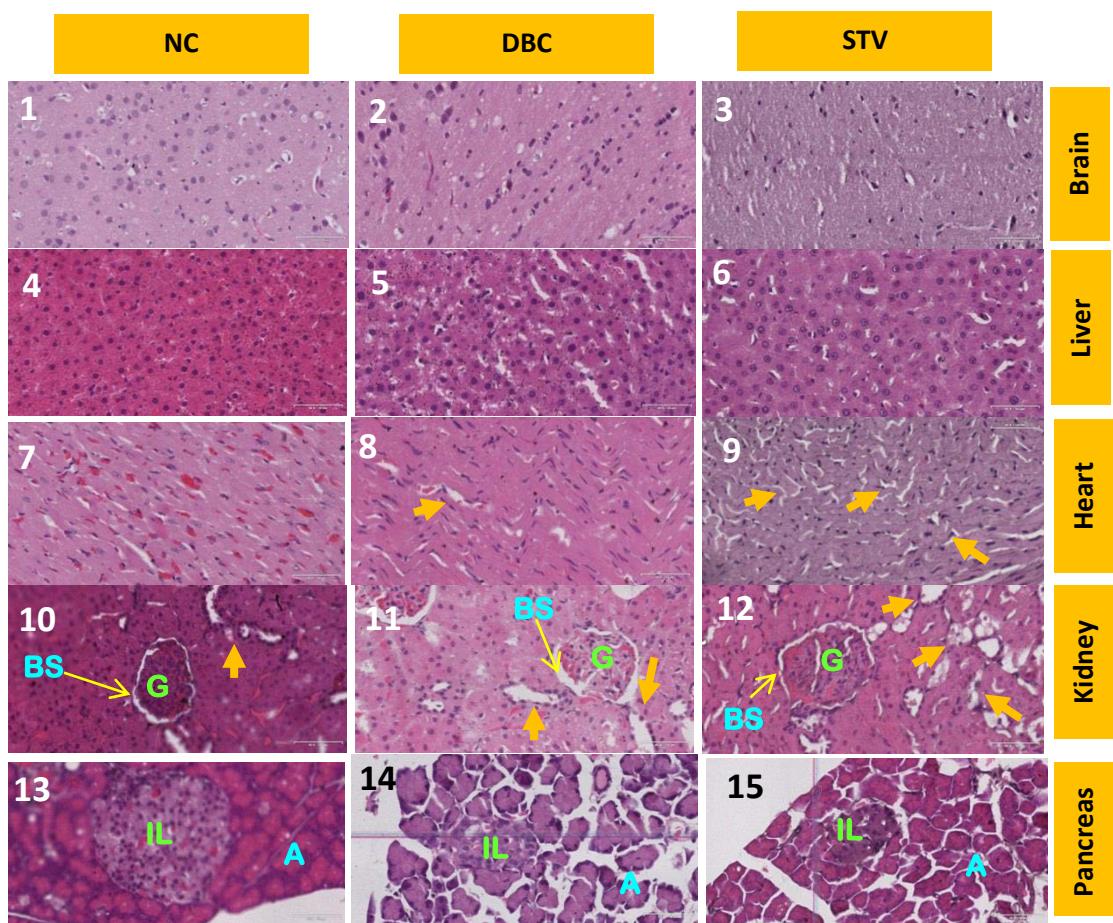


Figure 7.7: Histological examinations of the brain (1-3), liver (4-6), heart (7-9), kidney (10-12) and pancreas (13-15) of different animal groups at the end of the experimental period; at 400x total magnification. NC, Normal Control; DBC, Diabetic Control; STV, Stevia.

7.5 Discussion

Stevia is a naturally occurring NNS that has been shown in several studies to have antidiabetic properties (Sharma et al., 2012; Mohd-Radzaman et al., 2013; Shivanna et al., 2013). Interestingly, studies have used stevia in its pure form and not in the commercially available form which is consumed by people. The effects of the long-term uncontrolled consumption of stevia-based sweeteners on different organ histopathology and organ specific blood parameters have also not been studied in the previous studies. In the present study, we investigated the effects of a commercially available stevia-based NNS in an experimentally induced rat model of T2D. Although the supplementation of stevia-based NNS in drinking water maintained the weekly blood glucose at a relatively better concentration and ameliorated the histological changes caused by diabetes in the liver and pancreas, the treated rats displayed some heart injury with kidney vacuolation concomitant with elevated serum AST, LDH and CK-MB concentrations.

Diabetes is a disease characterized by chronic hyperglycemia which is marked by a number of symptoms including: weight loss, polydipsia, polyuria, and sometimes polyphagia and impaired vision (ADA, 2013). In the present study, induction of diabetes s increased food and fluid intake (Figure 7.1) and weekly blood glucose (Figure 7.3), and reduced body weight (Figure 7.2) when *ad libitum* supplementation of stevia did not significantly affect the above-mentioned parameters. Our results are in accordance with other studies which have reported that the consumption of stevia does not affect food intake and weight gain in normal and diabetic subjects (Munro et al., 2000; Figlewicz et al. 2009; Anton et al., 2010; Shivanna et al., 2013).

Glycemic control is critical in the management of T2D and uncontrolled hyperglycemia is strongly associated with diabetes-related complications (Fowler, 2008; Forbes and Cooper, 2013). A number recent and previous studies investigated the effects of either stevia extracts, extracted polyphenols, fibres or its pure glycosides such as stevia rebaudiana A, stevioside in different animal models of diabetes. In some previous studies, it has been reported that the daily oral administration of stevioside (0.5-25 mg/kg bw) for 15 d to 6 weeks significantly decreased blood glucose concentrations, improved insulin sensitivity, and improved glucose tolerance and pancreatic insulin content

respectively in experimentally induced type 1 and T2D rat model (Chen et al. 2005), fructose-fed insulin resistance rat model (Chang et al. 2005), spontaneously diabetic Goto Kakizaki rats (Jeppesen et al. 2003). In some very recent studies, the daily oral administration of another stevia glycoside, rebaudiana A, at the dose of 50-500 mg/kg bw significantly reduced fasting blood glucose and insulin resistance and protected β -cells in STZ-induced diabetic rats (Akbarzadeh et al. 2014; Saravanan et al. 2012) when 750 mg/kg bw shown toxicological effects in STZ-induced diabetic rats (Akbarzadeh et al. 2014). In another study, daily oral administration of rebaudiana A at a dose of 25 mg/kg bw for 8 weeks did not improve blood glucose, serum insulin and glucose tolerance in spontaneously diabetic Goto Kakizaki rats (Dyrskog et al. 2005). In our study, according to the calculation presented in Table 7.1, although the consumption of stevia glycoside (261.12 mg/kg bw) improved liver and pancreatic tissue damage and blood glucose concentrations (Fig. 7.7, Fig. 7.3), it did not show any significant anti-diabetic effect might be due to two reasons: (1) it was not administered as a single bolus dose like above-mentioned studies but at a very low concentration in water (0.0462%), (2) some of the anti-diabetic effects of stevia glycodises were might be compromised by its calorie containing additives, which might affect other parameters as well such as serum lipid profile.

Abnormal lipid profile and various organ function-related parameters along with uncontrolled diabetes can ultimately lead to the development of chronic diabetic complications such as- diabetic nephropathy; retinopathy; peripheral neuropathy; and autonomic neuropathy with the risk of cardiovascular dysfunction (Fowler, 2008; Forbes and Cooper, 2013). In a recent study, it has been reported that dietary supplementation of 4% stevia power or equivalent polyphenol reduced serum AST and ALT concentrations in diabetic but increased in non-diabetic rats when no effect of stevia fibre was observed on these parameters either in diabetic or normal rats (Shivanna et al. 2013). In a more recent study, Akbarzadesh et al. (2014) reported that daily oral administration of stevia rebaudiana A (250, 500 and 750 mg/kg bw) significantly reduced serum triglycerides but no effects were observed in serum total- and HDL-cholesterol. Although results were lower compared to the control, the serum ALP concentrations were dose-dependently higher in the stevia treated groups and the results of AST were not presented at all in this article. Liver damage was evident with higher level

of FBG and TG in the high (750 mg/kg bw) stevia treated group compared to the groups treated with lower dosages (250 and 500 mg/kg bw). Although a couple of recent studies reported the beneficial effects of stevia extracts in improving diabetes-induced kidney damage and kidney functions in rats, their animal models (STZ-nicotinamed and STZ-induced models vs fructose-STZ model) and duration of intervention (15 days and 4 weeks vs 13 week) were completely different from us (Ozbayer et al. 2011, Shivanna et al. 2013). Additionally, Melis (1995) reported that intake of stevia at a very high concentration can have detrimental histological effects on the kidney. In our study, although serum triglyceride, ALT and ALP and bowman space of kidney tissue were markedly reduced (Fig. 7.6, Table 7.2, Fig. 7.7), the significantly higher serum AST, LDH and CK-MB and marked increased serum uric acid concentrations (Table 7.2, Fig. 7.5) are in line with the cardiac and kidney tissue damage in stevia consuming group compared to the DBC group (Fig. 7.7). Results of our study are also supported by the results of the above-mentioned study.

In summary, although a number of previous studies reported the anti-diabetic effects of stevia extract and stevia glycosides in various experimental animal models, the duration of studies were shorter than our study (15 days to 8 weeks vs 13 week) and none of them used commercially available stevia-based NNS. In most of these studies, stevia was supplied either as a single oral dose or with diet when we have supplied with drinking water. It is important to note that the commercially available stevia used in the current study contains other biologically active ingredients, such as lactose which has been reported to have nutrient absorption enhancing activity (Kwak et al., 2012) as well as total calorie intake. Additionally, the total consumption of stevia glycoside per day per kg of body was significantly higher than the FDA approved acceptable daily intake (ADI). These factors may limit stevia's anti-diabetic potential and contribute to some of its toxic effects.

7.6 Conclusion

In conclusion, data of this study suggest that uncontrolled consumption commercially available stevia-based NNS at 10% sucrose equivalent dose has no significant anti-diabetic effects but have some toxicological effects in a T2D model of rats. Hence, diabetic individuals consuming commercially

available stevia-based NNS are suggested to limit their daily intake to avoid the possible toxicological effects.

7.7 References

Please refer to the reference section at the end of this thesis (pages 126-144).

CHAPTER EIGHT

General discussion and conclusion

8.1 General discussion

Although several studies have been conducted to investigate the safety of each NNS that we studied, the data are conflicting. Additionally, majority of studies have used NNSs in their pure form and not in the commercially available form which is consumed by people. Thus, the primary aim of the current study was to assess the effects of commercially available NNSs in an experimentally induced rat model of T2D. The results of this study indicate that uncontrolled consumption of commercially available NNSs for 13 week may influence some of the diabetes related parameters; such as serum lipid profile, organ histopathology and serum inflammatory biomarkers. Thus, individuals consuming NNSs are advised to limit their daily intake and check their lipid profile and inflammatory biomarkers in the serum.

Although some previous animal studies have suggested that intake of NNSs may promote weight gain, either by increasing energy intake (Swithers et al., 2013) or by decreasing energy expenditure (Feijó et al., 2013); the results of the present study indicates that consumption of NNSs does not influence food intake and weight gain. It is important to note that some of the previous studies showed increased weight gain compared to control group that was fed a high calorie sweetener such as glucose or sucrose (Swithers et al., 2009; Swithers et al., 2010; Swithers et al., 2012). It has been reported that pre-meal consumption of high calories leads to reduce food intake, a process known as caloric compensation (Allison, 2013). Thus, it is possible that the controls fed high calorie sugars were subjected to caloric compensation and consumed less food and this led to reduced weight gain when compared to treated groups. In support of this hypothesis, we recently conducted a pilot study in our lab, whereby normal rats were fed commercially available NNSs and both water and sucrose were used as control groups (Unpublished data). The results revealed that administration of commercially available NNSs to normal rats causes significantly increased weight gain only when compared to the sucrose control, and no difference was observed when comparing to the water control (Unpublished

data). Thus, no significant difference was observed in the present study because the control groups were administered with normal drinking water.

Even though, majority of the commercially available NNSs used in the present study did not influence glucose homeostasis in both diabetic (low dose) and normal (toxicological dose) rats; it was revealed that treatment with most of the commercially available NNSs led to increased total- and LDL-cholesterol and decreased triglycerides in the serum. A very recent study by Suez et al. (2014) revealed that treatment with NNSs leads to significant alterations of the gut microbiota. Other previous animal studies have shown that gut microbiota modulates host energy and lipid metabolism in mice (Velagapudi et al., 2010). Thus, intake of the commercially available NNSs may have altered the microbial composition in the gut of the rats, and ultimately favoured the absorption of dietary cholesterol and the increase in serum total and LDL-cholesterol. Further research is required to confirm this hypothesis.

Moreover, the results showed that administration of the NNSs ad libitum for 13 week may induce tissue damage, which was corroborated with increased inflammatory biomarkers in the serum. Although some studies have reported that NNSs are biologically inert compounds, other studies have shown that NNSs can cause significant changes in the body by altering gut microbiota (Suez et al., 2014), converting into more toxic compounds (Buss et al., 1992; Renwick et al., 2004), increasing common metabolites to toxic levels (Christian et al., 2004) or altering glucose regulating hormones (Swithers et al., 2012). Most of these studies used NNSs in their pure form, whereas in the present study NNSs were used in their commercially available form. The commercially available NNSs contain other biologically active ingredients which may influence the metabolic parameters, particularly organ damage.

One of the active ingredients that commercially available NNSs contain is lactose. Lactose has been previously shown to enhance the absorption of minerals, such as calcium in the colon of rats (Kwak et al., 2012). Thus, there is a possibility that lactose enhanced the absorption of the NNSs which led to their accumulation in different tissues. Accumulation of these NNSs could have caused tissue damage

and ultimately leakage of inflammatory biomarkers into the serum. Further studies are required to confirm this hypothesis.

8.2 General conclusion

The results of the present study suggest that intake of commercially available NNSs significantly influence some of the diabetes related parameters. Table 8.1 in the results section compares the effects of each NNS on the different diabetes related parameters. The table shows that sucralose has the most detrimental effects. Although stevia showed the lowest score, it cannot be stated that it holds the most beneficial effects since toxicological has not yet been conducted for this NNS. . Since the NNSs mostly cause tissue damage which is accompanied by increased inflammatory biomarkers in the serum, as well as lipid profile, individuals consuming commercially available sweeteners are advised to limit their daily intake, and check their lipid profile and inflammatory biomarkers in the serum. At the same time, stevia can be considered as the safer NNS compared to all other sweeteners used in this study.

8.3 Limitations and future studies

There are several limitations in the present study. Although it could be better to use either sucrose as a positive control group, the diabetic groups would have died due adverse hyperglycemia and its related complications before the end of the study period. Hence, it has not been included. Moreover, insulin tolerance test, glucose clearance rates and insulin sensitivity tests could add additional information to the study. Assessment of the quantity of each NNS in the sugar substitutes and in the blood of rats could also add substantial value to the study.

Thus, future studies will be conducted and these include: quantification of each NNS using high pressure liquid chromatography and assessment of the presence or accumulation of NNSs in the different organs.

Table 8.1: Comparing the effects of each NNS in the diabetes and toxicity studies

	Aspartame		Sucratose		Cyclamate		Saccharin		Stevia
Study	Diabetes	Toxicity	Diabetes	Toxicity	Diabetes	Toxicity	Diabetes	Toxicity	Diabetes
Fluid intake				↑				↑	
3h-FBG									↓↓
Total Cholesterol			↑						
HDL Cholesterol				↓				↓	
LDL Cholesterol	↑		↑	↑		↑		↑	
Triglycerides						↓		↓	
Alkaline Phosphatase			↑↑				↑↑		
Alanine Transaminase					↑↑	↓		↓	

Aspartate Transaminase	↑								↑
Lactate Dehydrogenase	↑		↑		↑			↑	↑
Creatine Kinase	↑		↑						↑
Fructosamine		↓		↓		↓	↓↓	↓	
Uric Acid			↑↑						
Creatinine		↓							
Brain Tissue Damage	↑↑↑			↑↑					
Liver Tissue Damage	↑	↑↑		↑	↑↑↑	↑↑	↑		↓
Heart Tissue Damage	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑			↑↑	

Kidney Tissue Damage	↑	↑↑↑	↑	↑↑	↑↑			↑↑	↑
Pancreas Tissue Damage	↓		↑↑	↑	↑↑	↑	↑		↓
Score in each Study	+11	+8	+14	+11	+10	+2	+2	+3	+2
Average Score	+9.5		+12.5		+6		+2.5		+2

Note: Red arrow represents detrimental effect and, whilst green arrow represents beneficial effect. Each red arrow scores +1 and each green arrow scores -1. In the diabetes study, one arrow was given for each significant difference ($p < 0.05$) when compared to the NC group, when DBC did cause a significant change; two arrows were given when the change was significantly different ($p < 0.05$) when compared to the DBC group. For the toxicity study, one arrow was given for each significant difference ($p < 0.05$), when compared to the NC group. Tissue damages were scored based on the severity of damage compared to NC (for toxicity) and DBC (for diabetes) groups.

References

- Abhilash M, Paul MVS, Verghese MV, Nair RH. Effect of Long Term Intake of Aspartame on Antioxidant Defense in Liver. *Food and Chemical Toxicology* 2011; 49: 1203-1207
- Abou-Seif M.A, Youssef A. Evaluation of Some Biochemical Changes in Diabetic Patients. *Clinica Chimica Acta* 2004; 346: 161-170
- Abudula R, Jeppesen PB, Rolfsen SE, Xiao J, Kermansen K. Rebaudioside A Potently Stimulates Insulin Secretion from Isolated Mouse Islets: Studies on the Dose-, Glucose-, and Calcium-Dependency. *Metabolism* 2004; 53: 1378-1381
- Ajala O, English P, Pinkney J. Systematic Review and Meta-Analysis of Different Dietary Approaches to the Management of Type 2 Diabetes. *American Journal of Clinical Nutrition* 2013; 97: 505-516
- Allison DB. Liquid Calories, Energy Compensation and Weight: What We Know and What We Still Need to Learn. *British Journal of Nutrition* 2014; 111: 384-386
- American Diabetes Association (ADA): Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 2007; 30: S42-S47
- American Diabetes Association. Standards of Medical Care in Diabetes. *The Journal of Clinical and Applied Research and Education* 2015; 38: S1-S94
- American Diabetes Association. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 2013; 36: S67-S74
- American Diabetes Association. Dyslipidemia Management in Adults with Diabetes. *Diabetes Care* 2004; 27: S68-S71
- Amo K, Arai H, Uebanso T, Fukaya M, Koganei M, Sasaki H et al. Effects of Xylitol on Metabolic Parameters and Visceral Fat Accumulation. *Journal of Clinical Biochemistry and Nutrition* 2011; 49: 1-7
- Amod A, Riback W, Schoeman HS. Diabetes Guidelines and Clinical Practice: Is there a Gap? The South African Cohort of the International Diabetes Management Practices Study. *The Journal of Endocrinology, Metabolism and Diabetes of South Africa* 2012; 17: 85-90

Andrejic BM, Mijatovic VM, Samojlik IN, Horvat OJ, Calasan JD, Dolai MA. The Influence of Chronic Intake of Saccharin on Rat Hepatic and Pancreatic Function and Morphology: Gender Differences. *Bosnian Journal of Basic Medical Sciences* 2013; 13: 94-99

Andrejić BM, Mijatović VM, Samojlik IN, Horvat OJ, Čalasan JD, Dolai MA. The Influence of Chronic Intake of Saccharin on Rat Hepatic and β Function and Morphology: Gender Differences. *Bosnian Journal of Basic Medical Science* 2013; 13: 94-99

Anton SD, Martin CK, Han H, Coulon S, Cefalu WT, Geiselman P, Williamson DA. Effects of Stevia, Aspartame, and Sucrose on Food Intake, Satiety, and Postprandial Glucose and Insulin Levels. *Appetite* 2010; 55: 37-43

Aronson KJ, Miller AB, Woolcott CG. Breast Adipose Tissue Concentrations of Polychlorinated Biphenyls and Other Organochlorines and Breast Cancer Risk. *Cancer Epidemiology, Biomarkers and Prevention* 2000; 9: 55-63.

Ashcroft FM, Rorsman P. Diabetes Mellitus and the β Cell: The Last Ten Years. *Cell* 2012; 148: 1160-1171

Ashok I, Sheeladevi R, Wankhar D. Acute Effect of Aspartame-Induced Oxidative Stress in Wistar Albino Rat Brain. *The Journal of Biomedical Research* 2014; 28: 1-7

Ayyappa B, Kanchi S, Singh P, Sabela MI, Dovey M, Bisetty. Analytical Evaluation of Steviol Glycosides by Capillary Electrophoresis Supported with Molecular Docking Studies. *Journal of the Iranian Chemical Society* 2014; DOI 10.1007/s13738-014-0465-z

Baird IM, Shephard NW, Merritt RJ, Hildick-Smith G. Repeated Dose Study of Sucratose Tolerance in Human Subjects. *Food and Chemical Toxicology* 2000; 38: S123-129

Benton D. Can Artificial Sweeteners Help Control Body Weight and Prevent Obesity? *Nutrition Research Reviews* 2005; 18: 63-76

Bergstrom BP, Cummings DR, Skaggs TA. Aspartame Decreases Evoked Extracellular Dopamine Levels in the Rat Brain: An In Vivo Voltammetry Study. *Neuropharmacology* 2007; 53: 967-974

Bertram MY, Jaswal AVS, Van Wyk VP, Levitt NS, Hofman KJ. The Non-Fatal Disease Burden Caused by Type 2 Diabetes in South Africa, 2009. *Global Health Action* 2013; 6: 206-212

Beverage Institute for Health and Wellness: Available at:
<http://beverageinstitute.org/article/stevia-sweeteners-safety-adi-and-metabolism/> (Last accessed: 13 November 2014)

Binns NM. Sucralose-All Sweetness and Light. *British Nutrition Foundation Nutrition Bulletin* 2003; 28: 53-58

Boesten DMPHJ, Berger A, de Cork Peter, Dong Hau, Hammock BD, Hartog GJM, Bast A. Multi-Targeted Mechanisms Underlying the Endothelial Protective Effects of the Diabetic-Safe Sweetener Erythritol. *Plos One* 2013; 8: e65741-e65741

Brown AW, Brown MMB, Onken KL, Beitz DC. Short-term Consumption of Sucralose, a Nonnutritive Sweetener, is Similar to Water with Regard to Selected Markers of Hunger Signaling and Short-term Glucose Homeostasis in Women. *Nutrition Research* 2011; 31: 882-888

Brown RJ, De Banate MA, Rother KI. Artificial Sweeteners: A Systematic Review of Metabolic Effects in Youth. *International Journal of Pediatric Obesity* 2010; 5: 305-312

Brown RJ, Rother KI. Non-nutritive Sweeteners and their Role in the Gastrointestinal Tract. *The Journal of Clinical Endocrinology and Metabolism* 2012; 97: 2597-2605

Bryant CE, Wasse LK, Astbury N, Nandra G, McLaughlin JT. Non-Nutritive Sweeteners: No Class Effect on the Glycaemic or Appetite Responses to Ingested Glucose. *European Journal of Clinical Nutrition* 2014; 68: 629-631

Buchanan TA. Pancreatic Beta-Cell Loss and Preservation in Type 2 Diabetes. *Clinical Therapeutics* 2003; 25: B32-B46

Burkitt DP, Walker ARP, Painter NS. Effect of Dietary Fibre on Stools and Transit Times, and its Role in the Causation of Disease. *Lancet* 1972; 2: 1408-1412

Buss NE, Renwick AG, Donaldson KM, George CF. The Metabolism of Cyclamate to Cyclohexylamine and its Cardiovascular Consequences in Human Volunteers. *Toxicology and Applied Pharmacology* 1992; 115: 199-210

Butchko HH, Stargel WW, Comer CP, Mayhew DA, Benninger C, Blackburn GL et al. Aspartame: Review of Safety. *Regulatory Toxicology and Pharmacology* 2002; 35: S1-S93

Canderel website: www.canderel.co.za/our-range (Last visited: 29 September, 2014)

Carlson HE, Jayendra HS. Aspartame and its Constituent Amino Acids: Effects on Prolactin, Cortisol, Growth Hormone, Insulin, and Glucose in Normal Humans. *The American Journal of Clinical Nutrition* 1989; 49: 427-432

Celik I, Yegin E, Odabasoglu F. Effect of Experimental Diabetes Mellitus on Plasma Lactate Dehydrogenase and Glutamic Oxaloacetic Aminotransferase Levels in Rabbits. *Turkish Journal of Biology* 2002; 26: 151-154

Cerf ME. Beta Cell Dysfunction and Insulin Resistance. *Frontiers in Endocrinology* 2013; 4: 1-12

Chang JC, Wu MC, Liu IM, Cheng JT. Increase of Insulin Sensitivity by Stevioside in Fructose-Rich Chow-Fed Rats. *Hormone and Metabolic Research* 2005; 37: 610-616.

Chen L, Magliano DJ, Zimmet PZ. The Worldwide Epidemiology of Type 2 Diabetes Mellitus-Present and Future Perspectives. *Endocrinology* 2011; 8: 228-236

Chen X, Zheng Y, Shen Y. Voglibose (Basen, AO-128), One of the Most Important Alpha-Glucosidase Inhibitors. *Current Medicinal Chemistry* 2006; 13: 106-116

Chow BF, Meier P, Free SM. Absorption of Vitamin B₁₂ Enhanced by D-Sorbitol. *The American Journal of Clinical Nutrition* 1958; 6: 30-33

Christian B, McConnaughey K, Bethea E, Brantley S, Coffey A, Hammond L, Harrell S, Metcalf K, Muehlenbein D, Spruill W, Brinson L, McConnaughey M. Chronic Aspartame Affects T-maze Performance, Brain Cholinergic Receptors and Na⁺, K⁺-ATPase in Rats. *Pharmacology, Biochemistry and Behavior* 2004; 78: 121-127

Clark M. Lifestyle Self-Management in Patients with Type 2 Diabetes. *Journal of Diabetes Nursing* 2002; 6: 182-187

Cohen SM, Arnold LL, Emerson JL. Safety of Saccharin. *Agro Food Industry Hi Tech Archive* 2008; 19: 24-27

Collison KS, Makhoul NJ, Zaidi MZ, Al-Rabiah R, Iglesias A., Andres BL, Ubungen R, Shoukri M, Al-Mohanna FA. Interactive Effects of Neonatal Exposure to Monosodium Glutamate and Aspartame on Glucose Homeostasis. *Nutrition and Metabolism* 2012a; 9: 58-70

Collison KS, Makhoul NJ, Zaidi MZ, Saleh SM, Andres B, Inglesias A, Al-Rabiah R, Al-Mohanna FA. Gender Dimorphism in Aspartame-Induced Impairment of Spatial Cognition and Insulin Sensitivity. *PLoS ONE* 2012b; 7:1-13

Creasy DM, Ford GR, Gray TJB. The Morphogenesis of Cyclohexylamine-Induced Testicular Atrophy in the Rat: *In Vivo* and *In Vitro* Studies. *Experimental and Molecular Pathology* 1990; 52: 155-169

Del Guerra S, Lupi R, Marselli L, Masini M, Bugiani M, Sbrana S *et al.* Functional and Molecular Defects of Pancreatic Islets in Human Type 2 Diabetes. *Diabetes* 2006; 54: 727-735

Den Hartog GJM, Boots AW, Adam-Perrot A, Brouns F, Verkooijen IWCM, Weseler AR *et al.* Erythritol is a Sweet Antioxidant. *Nutrition* 2010; 26: 449-458

Dhingra R, Sullivan L, Jacques PF, Wang TJ, Fox CS, Meigs JB, D'Agostino RB, Gaziano JM, Vasan RS. Soft Drink Consumption and Risk of Developing Cardiometabolic Risk Factors and the Metabolic Syndrome in Middle-aged Adults in the Community. *Circulation* 2007; 116: e557

Dills WL. Sugar Alcohols as Bulk Sweeteners. *Annual Review of Nutrition* 1989; 9: 161-186

Dyrskog SE, Jeppesen PB, Chen J, Christensen LP, Hermansen K. The Diterpene Glycoside, Rebaudioside A, does not Improve Glycemic Control or Affect Blood Pressure after Eight Weeks Treatment in the Goto-Kakizaki Rat. *The Review of Diabetic Studies* 2005; 2: 84-91

Feijó FM, Ballard CR, Foletto KC, Batista BAM, Neves AM, Ribeiro MFM, Bertoluci MC. Saccharin and Aspartame, Compared with Sucrose, Induce Greater Weight Gain in Adult Wistar Rats, at Similar Total Caloric Intake Levels. *Appetite* 2013; 60: 203-207

Figlewicz DP, Ioannou G, Jay JB, Kittleson S, Savard C, Roth CL. Effect of Moderate Intake of Sweeteners on Metabolic Health in the Rat. *Physiology and Behavior* 2009; 98: 618-624

Fimognari FL, Pastorelli R, Incalzi RA. Phenformin-Induced Lactic Acidosis in an Older Diabetic Patient. *Diabetes Care* 2006; 29: 950-951

Florez JC. Newly Identified Loci Highlight Beta Cell Dysfunction as a Key Cause of Type 2 Diabetes: Where are the Insulin Resistance Genes? *Diabetologia* 2008; 51: 1100-1110

Food and Drug Administration (FDA). *Federal Register* 1998; 63: 16417-16433

Forbes JM, Cooper ME. Mechanisms of Diabetic Complications. *Physiology Reviews* 2013; 93: 137-188

Ford HE, Peters V, Martin NM, Sleeth ML, Ghatei MA, Frost GS, Bloom SR. Effects of Oral Ingestion of Sucralose on Gut Hormone Response and Appetite in Healthy Normal-weight Subjects. *European Journal of Clinical Nutrition* 2011; 65: 508-513

Fowler MJ. Microvascular and Macrovascular Complications of Diabetes. *Clinical Diabetes* 2008; 26: 77-82

Gallus S, Scotti L, Negri E, Talamini R, Franceschi S, Montella M, Giacosa A, Dal Maso L, La Vecchia C. Artificial Sweeteners and Cancer Risk in a Network of Case-Control Studies. *Annals of Oncology* 2007; 18: 40-44

Gardner C, Wylie-Rosett J, Gidding SS, Steffen LM, Johnson RK, Reader D et al. Nonnutritive Sweeteners: Current Use and Health Perspectives. A Scientific Statement from the American Heart Association and the American Diabetes Association. *Circulation* 2012; 126: 509-519

Gauriguata L, Whiting DR, Hambleton I, Beagley J, Linnenkamp U, Shaw JE. Global Estimates of Diabetes Prevalence for 2013 and Projections for 2035. *Diabetes Research and Clinical Practice* 2014; 103: 137-149

Ghosh S, Sudha ML. A Review on Polyols: New Frontiers for Health-Based Bakery Products. *International Journal of Food Sciences and Nutrition* 2012; 63: 372-379

Gostner A, Schaffer V, Theis S, Menzel T, Luhrs H, Melcher R. Effects of Isomalt Consumption on Gastrointestinal and Metabolic Parameters in Healthy Volunteers. *British Journal of Nutrition* 2005; 94: 575-581

Gougeon R, Spidel M, Lee K, Field CJ. Canadian Diabetes Association National Nutrition Committee Technical Review: Non-Nutritive Intense Sweeteners in Diabetes Management. *Canadian Journal of Diabetes* 2004; 28: 385-399

Gregersen S, Jeppesen PB, Holst JJ, Hermansen K. Antihyperglycemic Effects of Stevioside in Type 2 Diabetic Subjects. *Metabolism* 2004; 53: 73-76.

Griffith ML, Younk LM, Davis SN. Visceral Adiposity, Insulin Resistance, and Type 2 Diabetes. *American Journal of Lifestyle Medicine* 2010; 4: 230-243

Grotz VL, Henry RR, McGill JB, Prince MJ, Shamo H, Trout JR, Pi-Sunyer FX. Lack of Effect of Sucralose on Glucose Homeostasis in Subjects with Type 2 Diabetes. *Journal of the American Dietetic Association* 2003; 103: 1607-1612

Halldorsson TI, Strom M, Peterson SB, Olsen SF. Intake of Artificially Sweetened Soft Drinks and Risk of Preterm Delivery: A Prospective Cohort Study in 59,334 Danish Pregnant Women. *American Journal of Clinical Nutrition* 2010; 92: 626-633

Harita N, Hayashi T, Sato KK, Nakamura Y, Yoneda T, Endo G, Kambe H. Lower Serum Creatinine is a New Risk Factor of Type 2 Diabetes: The Kansai Healthcare Study. *Diabetes Care* 2009; 32: 424-426

Harris EH. Elevated Liver Function Tests in Type 2 Diabetes. *Clinical Diabetes* 2005; 23: 115-119

Hashemi SA, Allameh A, Daraei B, Peynevandi KM, Pashazadeh R. Effect of Rebadioside A on Attenuation of Oxidative Stress in Kidney of Mice under Acetaminophen Toxicity. *Iranian Journal of Toxicology* 2013; 7: 944-951

Hauner H. The Mode of Action of Thiazolidinediones. *Diabetes/Metabolism Research and Reviews* 2002; 18: S10-S15

Hayes C. The Effect of Non-Cariogenic Sweeteners on the Prevention of Dental Caries: A Review of the Evidence. *Journal of Dental Education* 2001; 65: 1106-1109

Hoffmann J, Spengler M. Efficacy of 24-Week Monotherapy with Acarbose, Metformin or Placebo in Dietary-Treated NIDDM Patients: The Essen-II Study. *The American Journal of Medicine* 1997; 103: 483-490

Hu FB, Malik VS. Sugar-sweetened Beverages and Risk of Obesity and Type 2 Diabetes: Epidemiologic Evidence. *Physiology and Behavior* 2010; 100: 47-54

Hunty A, Gibson S, Ashwell. A Review of the Effectiveness of Aspartame in Helping with Weight Control. *British Nutrition Foundation* 2006; 31: 115-128

International Diabetes Federation (IDF). *IDF Diabetes Atlas*, 6th Edition 2013. International Diabetes Federation, Brussels, Belgium.

Islam MS, Indrajit M. Effects of Xylitol on Blood Glucose, Glucose Tolerance, Serum Insulin and Lipid Profile in a Type 2 Diabetes Model of Rats. *Annals of Nutrition and Metabolism* 2012; 61: 57-64

Islam MS, Sakaguchi E. Sorbitol-based Osmotic Diarrhea: Possible Causes and Mechanism of Prevention investigated in rats. *World Journal of Gastroenterology* 2006; 12: 7635-7641

Islam MS. Effects of Xylitol as a Sugar Substitute on Diabetes-Related Parameters in Nondiabetic Rats. *Journal of Medicinal Food* 2011; 14: 505-511

Jensen MD. Is Visceral Fat Involved in the Pathogenesis of the Metabolic Syndrome? Human Model. *Obesity* 2006; 14: 20S-24S

John BA, Wood SG, Hawkins DR. The Pharmacokinetics and Metabolism of Sucralose in the Mouse. *Food and Chemical Toxicology* 2000a; 38: S107-S110

John BA, Wood SG, Hawkins DR. The Pharmacokinetics and Metabolism of Sucralose in the Rabbit. *Food and Chemical Toxicology* 2000b; 38: S111-S113.

Johnson RJ, Segal MS, Sautin Y, Nakagawa T, Feig DI, Kang DH et al. Potential Role of Sugar (Fructose) in the Epidemic of Hypertension, Obesity and the Metabolic Syndrome, Diabetes, Kidney Disease, and Cardiovascular Disease. *The American Journal of Clinical Nutrition* 2007; 86: 899-906

Just T, Pau HW, Engel U, Hummel T. Cephalic Phase Insulin Release in Healthy Humans after Taste Stimulation? *Appetite* 2008; 51:622-627

Kadoglou NP, Iliadis F, Angelopoulou. The Anti-Inflammatory Effects of Exercise Training in Patients with Type 2 Diabetes Mellitus. *European Journal of Cardiovascular Prevention and Rehabilitation* 2007; 14: 837-843

Kalra S, Chadha M, Sharma SK, Unnikrishnan AG. Untapped Diamonds for Untamed Diabetes: The α -Glucosidase Inhibitors. *Indian Journal of Endocrinology and Metabolism* 2014; 18: 138-141

Kauffman GB, Priebe PM. The Discovery of Saccharin: A Centennial Retrospect. *Ambix* 1978; 25: 191-207

Kim JY, Seo J, Cho KH. Aspartame-Fed Zebrafish Exhibit Acute Deaths with Swimming Defects and Saccharin-Fed Zebrafish have Elevation of Cholesteryl Ester Transfer Protein Activity in Hypercholesterolemia. *Food and Chemical Toxicology* 2011; 49: 2899-2905

Kirk JK, Graves DE, Craven TE, Lipkin EW, Austin M, Margolis KL. Restricted-Carbohydrate Diets in Patients with Type 2 Diabetes: A Meta-Analysis. *Journal of American Dietetic Association* 2008; 108: 91-100

Kujur RS, Singh V, Ram M, Yadav HN, Singh KK, Kumari S, Roy BK. Antidiabetic Activity and Phytochemical Screening of Crude Extract of Stevia Rebaudiana in Alloxan-Induced Diabetic Rats. *Pharmacognosy Research* 2010; 2: 258-263

Kuzuya T, Nakagawa S, Satoh J, Kanazawa Y, Iwamoto Y, Kobayashi M et al. Report of the Committee on the Classification and Diagnostic Criteria of Diabetes Mellitus. *Diabetes Research and Clinical Practice* 2002; 55: 65-85

Kwak HS, Lee WJ, Lee MR. Revisiting Lactose as an Enhancer of Calcium Absorption. *International Dairy Journal* 2012; 22: 147-151

Lau K, McLean WG, Williams DP, Howard CV. Synergistic Interactions between Commonly Used Food Additives in a Developmental Neurotoxicity Test. *Toxicological Sciences* 2006; 90: 178-187

Lehmann JM, More LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA. An Antidiabetic Thiazolidinedione is a High Affinity Ligand for Peroxisome Proliferator-Activated Receptor Gamma (PPAR Gamma). *The Journal of Biological Chemistry* 1995; 270: 12953-12956

Lipinski GWR. Sweeteners. *Advanced Biochemical Engineering and Biotechnology* 2013; 10: 222-249

Livesey G, Buss D, Coussette P, Edwards DG, Howlett J, Jonas DA et al. Suitability of Traditional Energy Values for Novel Foods and Food Ingredients. *Food Control* 2000; 11: 249-289

Livesey G. The Energy Values of Dietary Fibre and Sugar Alcohols for Man. *Nutrition Research Reviews* 1992; 5: 61-84

Loria A, Medal LS, Elizondo J. Effect of Sorbitol on Iron Absorption in Man. *The American Journal of Clinical Nutrition* 1962; 10: 124-127

Ludwig DS, Peterson KE, Gortmaker SL. Relation between Consumption of Sugar-Sweetened Drinks and Childhood Obesity: A Prospective, Observational Analysis. *Lancet* 2001; 357: 505-508

Ma J, Chang J, Checklin HL, Young RL, Jones KL, Horowitz M et al. Effect of the Artificial Sweetener, Sucratose, on Small Intestinal Glucose Absorption in Healthy Human Subjects. *British Journal of Nutrition* 2010; 104: 803-806

Magnuson BA, Burdock GA, Doull J, Kroes RM, Marsh GM, Pariza MW et al. Aspartame: A Safety Evaluation based on Current use Levels, Regulations, and Toxicological and Epidemiological Studies. *Critical Reviews in Toxicology* 2007; 37: 629-727

Malik VS, Popkin BM, Bray GA, Despres J-P, Willett WC, Hu FB. Sugar-Sweetened Beverages and Risk of Metabolic Syndrome and Type 2 Diabetes. *Diabetes Care* 2010; 33: 2477-2483

Malik VS, Willett WC, Hu FB. Sugar-Sweetened Beverages and BMI in Children and Adolescents: Reanalysis of Meta-Analysis. *American Journal of Clinical Nutrition* 2009 89: 238-439

Mann SW, Yuschkak MM, Amyes SJ, Aughton P, Finn JP. A Combined Chronic Toxicity/Carcinogenicity Study of Sucralose in Sprague-Dawley Rats. *Journal of Food and Chemical Toxicology* 2000; 38: S71-S89

Marteau P, Flourie B. Tolerance to Low-Digestible Carbohydrates: Symptomatology and Methods. *British Journal of Nutrition* 85: S17-S21

Martins AT, Azoubel R, Lopes RA, Sala di Matteo MA, Ferraz de Arruda JG. Effect of Sodium Cyclamate on the Rat Fetal Liver: A Karyometric and Stereological Study. *International Journal of Morphology* 2005; 23: 221-226

Matikainen N, Taskinen M-R. Management of Dyslipidemias in the Presence of the Metabolic Syndrome or Type 2 Diabetes. *Current Cardiology Reports* 2012; 14: 721-731

Meigs JB, Muller DC, Nathan DM, Blake DR, Andres R. The Natural History of Progression from Normal Glucose Tolerance to Type 2 Diabetes in the Baltimore Longitudinal Study of Aging. *Diabetes* 2003; 52: 1475: 1484

Melis MS. Chronic Administration of Aqueous Extract of Stevia Rebaudiana in Rats: Renal Effects. *Journal of Ethnopharmacology* 1995; 47: 129-134

Melis MS. Effects of Chronic Administration of Stevia Rebaudiana on Fertility in Rats. *Journal of Ethnopharmacology* 1999; 67: 157-161

Miller RA, Chu Q, Xie J, Foretz M, Viollet B, Birnbaum M. Biguanides Suppress Hepatic Glucagon Signaling by Decreasing Production of Cyclic AMP. *Nature* 2013; 494: 256-260

Misra A, Singhal N, Khurana L. Obesity, the Metabolic Syndrome, and Type 2 Diabetes in Developing Countries: Role of Dietary Fats and Oils. *Journal of the American College of Nutrition* 2013; 29: 289S-301S

Misra H, Soni M, Silawat N, Mehta D, Mehta BK, Jain DC. Antidiabetic Activity of Medium-Polar Extract from the Leaves of Stevia Rebaudiana Bert. (Bertoni) on Alloxan-Induced Diabetic Rats. *Journal of Pharmacy and Biollied Sciences* 2011; 3: 242-248

Mitsutomi K, Masaki T, Shimasaki T, Gotoh K, Chiba S, Kakuma T, Shibata H. Effects of a Nonnutritive Sweetener on Body Adiposity and Energy Metabolism in Mice with Diet-Induced obesity. *Metabolism, Clinical and Experimental* 2014; 63: 69-78

Mohd-Radzman NH, Ismail WIW, Adam Z, Jaapar SS, Adam A. Potential Roles of Stevia Rebaudiana Bertoni in Abrogating Insulin Resistance and Diabetes: A Review. *Evidence-Based Complementary and Alternative Medicine* 2013; 2013: 1-11

Mohd-Radzman NH, Ismail WIW, Adam Z, Jaapar SS, Adam A. Potential Roles of Stevia Rebaudiana Bertoni in Abrogating Insulin Resistance and Diabetes: A Review. *Evidence-Based Complementary and Alternative Medicine* 2013; 2013: 1-11

Mooradian AD. Dyslipidemia in Type 2 Diabetes Mellitus. *Nature Clinical Practice Endocrinology and Metabolism* 2009; 5: 150-159

Munro PJ, Lurette A, Anderson DM, Ju HY. Effects of a New Sweetener, Stevia, on Performance of Newly Weaned Pigs. *Canadian Journal of Animal Science* 2000; 80: 529-531

Nakagawa T, Hu H, Zharikov S, Tuttle KR, Short RA, Glushakova O et al. A Casual Role for Uric Acid in Fructose-Induced Metabolic Syndrome. *The American Journal of Physiology. Renal Physiology* 2006; 290: F625-F631

Natural Health Research Institute. Available at:
<http://www.naturalhealthresearch.org/extrapolation-of-animal-dose-to-human/> (last accessed: 13 November, 2014)

Neacsu NA, Madar A. Artificial Sweeteners versus Natural Sweeteners. *Bulletin of Transilvania University of Brasov Series V: Economic Sciences* 2014; 56: 59-64

Negro F. Hepatotoxicity of Saccharin. *The New England Journal of Medicine* 1994; 331: 134-135

Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic Steatohepatitis: Summary of an AASLD Single Topic Conference on Hepatology 2003; 37: 1202-1219

Noda K, Nakayama K, Oku T. Serum Glucose and Insulin Levels and Erythritol Balance after Oral Administration of Erythritol in Health Subjects. *European Journal of Clinical Nutrition* 1994; 48: 286- 292

Nyenwe EA, Jerkins TW, Umpierrez GE, Kitabchi AE. Management of Type 2 Diabetes: Evolving Strategies for the Treatment of Patients with Type 2 Diabetes. *Metabolism Clinical and Experimental* 2011; 60: 1-23

Oku T, Okazaki M. Laxative Threshold of Sugar Alcohol Erythritol in Human Subjects. *Nutrition Research* 1996; 16: 577-589

Oliver RC, Tervonen T, Flynn DG, Keenan KM. Enzyme Activity in Crevicular Fluid in Relation to Metabolic Control of Diabetes and other Periodontal Risk Factors. *Journal of Periodontology* 1993; 91: 423-426

Oser BL, Carson S, Cox GE, Vogen EE, Sternberg SS. Long-Term and Multigeneration Toxicity Studies with Cyclohexylamine Hydrochloride. *Toxicology* 1976; 6: 47-65

Park J-S, Yoo SB, Kim JY, Lee SJ, Oh S-B, Kim J-S, et al. Effects of Saccharin Intake on Hippocampal and Cortical Plasticity in Juvenile and Adolescent Rats. *Korean Journal of Physiology and Pharmacology* 2010; 14: 113-118

Parthasarathy NJ, Kumar RS, Manikandan S, Devi RS. Methanol-induced Oxidative Stress in Rat Lymphoid Organs. *Journal of Occupational Health* 2006; 48: 20-27

Pernicova I, Korbonits M. Metformin- Mode of Action and Clinical Implications for Diabetes and Cancer. *Nature Reviews Endocrinology* 2014; 10: 143-156

Petrie JR, Pearson ER, Sutherland C. Implications of Genome Wide Association Studies for the Understanding of Type 2 Diabetes Pathophysiology. *Biochemical Pharmacology* 2011; 81: 471-477

Pettitt DJ, Talton J, Dabelea D, Divers J, Imperatore G, Lawrence JM et al. Prevalence of Diabetes in US Youth in 2009: The Search for Diabetes in Youth Study. *Diabetes Care* 2014; 37: 402-408

Plagnol V, Howson JMM, Smyth DJ, Walker N, Hafler JP, Wallace C et al. Genome-Wide Association Analysis of Autoantibody Positivity in Type 1 Diabetes Cases. *Plos Genetics* 2011; 7: 1-9

Pratt M, Lightowler H, Henry CJ, Thabuis C, Wils D, Guerin-Deremaux L. No Observable Differences in Glycemic Response to Maltitol in Human Subjects from 3 Ethnically Diverse Groups. *Nutrition Research* 2011; 31: 223-228

Price JM, Biava CG, Oser BL, Vogen EE, Steinfield J, Ley HL. Bladder Tumors in Rats Fed Cyclohexylamine or High Doses of a Mixture of Cyclamate and Saccharin. *Science* 167: 1131-1132

Quilez J, Bullo M, Salas-Salado J. Improved Postprandial Response and Feeling of Satiety after Consumption of Low-Calorie Muffins with Maltitol and High-Amylose Corn Starch. *Journal of Food Science* 2007; 72: S407-S411

Raben A, Vasilaras TH, Moller AC, Astrup A. Sucrose Compared with Artificial Sweeteners: different effects on *ad libitum* Food Intake and Body Weight after 10 Weeks of Supplementation in Overweight Subjects. *American Journal of Clinical Nutrition* 2002; 76: 721-729

Rahman MA, Islam MS. Xylitol Improves Pancreatic Islets Morphology to Ameliorate Type 2 Diabetes in Rats: A Dose Response Study. *Journal of Food Science* 2014; 79: H1436-H1442

Rencuzogullari E, Tuylu BA, Topaktas M, Ila HB, Kayraldziz A, Arslan M et al. Genotoxicity of Aspartame. *Drug and Chemical Toxicology* 2004; 27: 257-268

Rendell M. Advances in Diabetes for the Millennium: Drug Therapy of Type 2 Diabetes. *Medscape General Medicine* 2004; 6: 9-16

Renwick AG, Nordmann H. First European Conference on Aspartame: Putting Safety and Benefits into Perspective. Synopsis of Presentations and Conclusions. *Food and Chemical Toxicology* 2007; 45: 1308-1313

Renwick AG, Thompson JP, O'Shaughnessy M, Walter EJ. The Metabolism of Cyclamate to Cyclohexylamine in Humans during Long-Term Administration. *Toxicology and Applied Pharmacology* 2004; 196: 367-380

Ripsin CM, Kang H, Urban RJ. Management of Blood Glucose in Type 2 Diabetes Mellitus. *American Family Physician* 2009; 79: 29-36

Rizzo B, Zambonin L, Angeloni C, Leoncini E, Sega FVD, Prata C et al. Steviol Glycosides Modulate Glucose Transport in Different Cell Types. *Oxidative Medicine and Cellular Longevity* 2013; ID 348169: 1-12

Roberts A, Renwick AG, Sims J, Snodin DJ. Sucralose Metabolism and Pharmacokinetics in Man. *Food and Chemical Toxicology* 2000; 38: S31-S41

Rocha GS, Pereira MO, Benarroz MO, Frydman JN, Rocha VC, Pereira MJ, Fonseca AS, Medeiros AC, Bernardo-Filho M. Sucralose Sweetener In Vivo Effects on Blood Constituents

Radiolabeling, Red Blood Cell Morphology and Radiopharmaceutical Biodistribution in Rats. *Applied Radiation and Isotopes* 2011; 69: 46-51

Rodero AB, Rodero LS, Azoubel R. Toxicity of Sucralose in Humans: A Review. *International Journal of Morphology* 2009; 27: 239-244

Rosenbloom AL, Silverstein JH, Amemiya S, Zeitler P, Klingensmith GJ. Type 2 Diabetes Mellitus in the Child and Adolescent. *Pediatric Diabetes* 2008; 9: 512-526

Saada HN, Mekky NH, Eldawy HA, Abdelaal AF. Biological Effect of Sucralose in Diabetic Rats. *Food and Nutrition Sciences* 2013; 4: 82-89

Saha C, Racine FM. Biotechnological Production of Mannitol and its Application. *Applied Microbiology and Biotechnology* 2011; 89: 879-891

Saiki A, Miyashita Y, Wakabayasi K, Kameda N, Shirai K. Reduction of Visceral Adiposity after Operation in a Subject with Insulinoma. *Journal of Atherosclerosis Thombosis* 2004; 11: 209-214

Samuel VT, Shulman GI. Mechanisms for Insulin Resistance: Common Threads and Missing Links. *Cell* 2012; 148: 852-871

Saravanan R, Vengataswamy BK, Ramachandran V. Effect of Rebaudioside A, a Diterpenoid on Glucose Homeostasis in STZ-Induced Diabetic Rats. *Journal Physiology and Biochemistry* 2012; 68: 421-431

Sasaki YF, Kawaguchi S, Kamaya A, Ohshita M, Kabasawa K, Iwama K, Taniguchi K, Tsuda S. The Comet Assay with 8 Mouse Organs: Results with 39 currently used Food Additives. *Mutation Research* 2002; 519: 103-119

Savita SM, Sheela K, Sunanda S, Shankar AG, Ramakrishna P. Stevia rebaudiana- A Functional Component for Food Industry. *Journal of Human Ecology* 2004; 15: 261-264

Schiffman S.S., Rother K.I. Sucralose, a Synthetic Organochlorine Sweetener: Overview of Biological Issues. *Journal of Toxicology and Environmental Health* 2013, Part B; 16: 399-451

Schulze MB, Manson JE, Ludwig DS, Colditz GA, Stampfer MJ, Willet WC, Hu FB. Sugar-sweetened Beverages, Weight Gain, and Incidence of Type 2 Diabetes in Young and Middle-aged Women. *The Journal of American Medical Association* 2004; 292: 927-934

Scientific Committee on Food (SFC). Opinion on Erythritol. *European Commission: Health and Consumer Protection Directorate* 24 March 2003: <http://europa.eu.int/comm/food/fs/sc/scf>

Shaheen A, Khattak S, Khattak AZ, Kamal A, Jaffari SA, Sher A. Serum Alkaline Phosphatase Level in Patients with Type 2 Diabetes Mellitus and its Relation with Periodontitis. *KUST Medical Journal* 2009; 1: 51-54

Shankar P, Ahuja S, Sriram K. Non-Nutritive Sweeteners: Review and Update. *Nutrition* 2013; 29: 1293-1299

Sharma R, Yadav R, Manivannan E. Study of the Effect of Stevia Rebaudiana Bertoni on Oxidative Stress in Type-2 Diabetic Rat Models. *Biomedicine and Aging Pathology* 2012; 2: 126-131

Shawkat H, Westwood M-M, Mortimer A. Mannitol: A Review of its Clinical Uses. *Continuing Education in Anaesthesia, Critical Care and Pain* 2012; 12: 82-85

Shin YS, Park J, Kang DS, Yu J. Significance of the Measurement of Serum Fructosamine in the Management of Childhood Diabetes. *International Journal of Pediatric Endocrinology* 2013; 2013: 36

Shindou T, Sasaki Y, Miki H, Eguchi T, Hagiwara K, Ichikawa T. Identification of Erythritol by HPLC and GC-MS and Quantitative Measurement in Pulps of Various Fruits. *Journal of Agriculture, Food and Chemistry* 1989; 37: 1474-1476

Shivanna N, Naika M, Khanum F, Kaul VK. Antioxidant, Anti-diabetic and Renal Protective Properties of Stevia Rebaudiana. *Journal of Diabetes and Its Complication* 2013; 27: 103-113

Sims J, Roberts A, Danel JW, Renwick AG. The metabolic fate of sucralose in rats. *Food and Chemical Toxicology* 2000; 38: S115-S121

Singh Z. Toxicological Aspects of Saccharin. *Food Biology* 2013; 2: 4-7

Smith H. Oral Antihyperglycaemic Agents in Type 2 Diabetes Mellitus. *South African Pharmaceutical Journal* 2014; 81: 10-14

Society for Endocrinology, Metabolism and Diabetes of South Africa (SEMDSA). The 2012 SEMDSA Guidelines for the Management of Type 2 Diabetes. *Journal of Endocrinology, Metabolism and Diabetes of South Africa* 2012; 1-95

Soffritti M, Belpoggi F, Esposti DD, Lambertini L, Tibaldi E, Rigano A. First Experimental Demonstration of the Multipotential Carcinogenic Effects of Aspartame Administered in the Feed to Sprague-Dawley Rats. *Environmental Health Perspective* 2006; 114: 379-385

Stanhope KL, Havel PJ. Fructose Consumption: Recent Results and their Potential Implications. *Annals of the New York Academy of Sciences* 2010; 1190: 15-24

Stanhope KL, Schwarz JM, Keim NL, Griffen SC, Bremer AA, Graham JL et al. Consuming Fructose-sweetened, not Glucose-sweetened, Beverages Increases Visceral Adiposity and Lipids and Decrease Insulin Sensitivity in Overweight/Obese Humans. *The Journal of Clinical Investigation* 2009; 119: 1322-1334

Stanhope KL, Schwarz JM, Keim NL, Griffen SC, Bremer AA, Graham JL et al. Consuming Fructose-sweetened, not Glucose-sweetened, Beverages Increases Visceral Adiposity and Lipids and Decrease Insulin Sensitivity in Overweight/Obese Humans. *The Journal of Clinical Investigation* 2009; 119: 1322-1334

Storey D, Lee A, Bornet F, Brouns F. Gastrointestinal Tolerance of Erythritol and Xylitol Ingested in a Liquid. *European Journal of Clinical Nutrition* 2007; 61: 349-354

Suez J, Korem T, Zeevi D, Zilberman-Schapira G, Thaiss CA, Maza O et al. Artificial Sweeteners Induce Glucose Intolerance by Altering the Gut Microbiota. *Nature* 2014; In Press

Swithers SE, Baker CR, Davidson TL. General and Persistent Effects of High-Intensity Sweeteners on Body Weight Gain and Caloric Compensation in Rats. *Behavioral Neuroscience* 2009; 123: 772-780

Swithers SE, Davidson TL. A Role for Sweet Taste: Calorie Predictive Relations in Energy Regulation by Rats. *Behavioral Neuroscience* 2008; 122: 161-173

Swithers SE, Laboy AF, Clark K, Cooper S, Davidson TL. Experience with the High-Intensity Sweetener Saccharin Impairs Glucose Homeostasis and GLP-1 Release in Rats. *Behavioural Brain Research* 2012; 233: 1-14

Swithers SE, Martin AA, Clark KM, Laboy AF, Davidson TL. Body Weight Gain in Rats Consuming Sweetened Liquids. Effects of Caffeine and Diet Composition. *Appetite* 2010; 55: 528-533

Swithers SE, Sample CH, Davidson TL. Adverse Effects of High-Intensity Sweeteners on Energy Intake and Weight Control In Male and Obesity-Prone Female Rats. *Behavioral Neuroscience* 2013 127: 262-274

Sylvetsky AC, Welsh JA, Brown RJ, Vos MB. Low-Calorie Sweetener Consumption is increasing in the United States. *The American Journal of Clinical Nutrition* 2012; 96: 640-646

Takayama S, Renwick AG, Johansson SL, Thorgerisson UP, Tsutsumi M, Dalgard DW, Sierber SM. Long-Term Toxicity and Carcinogenicity Study of Cyclamate in Nonhuman Primates. *Toxicological Sciences* 2000; 53: 33-39

Tandel KR. Sugar Substitutes: Health Controversy over Perceived Benefits. *Journal of Pharmacology and Pharmacotherapy* 2011; 2: 236-243

Teff KL, Elliott SS, Tschop M, Kieffer TJ, Rader D, Heiman M et al. Dietary Fructose Reduces Circulating Insulin and Leptin, Attenuates Postprandial Suppression of Ghrelin, and Increases Triglycerides in Women. *The Journal of Clinical Endocrinology and Metabolism* 2004; 89: 2963-2972

The 2012 Society for Endocrinology, Metabolism and Diabetes of South Africa (SEMDSA) Guideline for the Management of Type 2 Diabetes. *Journal of Endocrinology, Metabolism and Diabetes of South Africa* 2012; 17: S1-S95

Thulé PM, Umpierrez G. Sulfonylureas: A New Look at Old Therapy. *Current Diabetes Reports* 2014; 14: 473-480

Touyz LZG. Saccharin Deemed “Not Harzadous” in the United States and Abroad. *Current Oncology* 2011; 18: 213-214

Turner RC, Millns H, Neil HAW, Stratton IM, Manley SE, Matthews DR, Holman RR. Risk factors for coronary artery disease in non-insulin dependent diabetes mellitus: United Kingdom prospective diabetes study (UKPDS: 23). *British Medical Journal* 1998; 316: 823-828

Turner RC, Millns H, Neil HAW, Stratton IM, Manley SE, Matthews DR, Holman RR. Risk Factors for Coronary Artery Disease in Non-Insulin Dependent Diabetes Mellitus: United Kingdom Prospective Diabetes Study (UKPDS: 23). *British Medical Journal* 1998; 316: 823-828

Van Belle TL, Coppieters KT, Von Herrath MG. Type 1 Diabetes: Etiology, Immunology, and Therapeutic Strategies. *Physiology Reviews* 2011; 91: 79-118

Velagapudi VR, Hezaveh R, Reigstad CS, Gopalacharyulu P, Yetukuri L, Islam S et al. The Gut Microbiota Modulates Host Energy and Lipid Metabolism in Mice. *Journal of Lipid Research* 2010 51: 101-1112

Verdonck LF, Sangster B, van Heijst AN, de Groot G, Maes RA. Buformin Concentrations in a Case of Fatal Lactic Acidosis. *Diabetologia* 1981; 20: 45-46

Weisbrod AV, Shea D, Moore MJ, Stegeman JJ. Species, Tissue and Gender-Related Organochlorine Bioaccumulation in White-Sided Dolphins, Pilot Whales and their Common Prey in the Northwest Atlantic. *Marine Environmental Research* 2001; 51: 29-50

Weyer C, Bogardus C, Mott DM, Pratley RE. The Natural History of Insulin Secretory Dysfunction and Insulin Resistance in the Pathogenesis of Type 2 Diabetes Mellitus. *Journal of Clinical Investigation* 1999; 104: 787-794

Whitehouse CR, Boullata J, McCauley LA. The Potential Toxicity of Artificial Sweeteners. *American Association of Occupational Health Nurses* 2008; 56: 251-259

Wilson RD, Islam MD. Fructose-fed Streptozotocin-injected Rat: An Alternative Model for type 2 Diabetes. *Pharmacological Reports* 2012; 64: 129-139

Winnick JJ, Sherman WM, Habash DL. Short-Term Aerobic Exercise Training in Obese Humans with Type 2 Diabetes Mellitus Improves Whole-Body Insulin Sensitivity though Gains in Peripheral, not Hepatic Insulin Sensitivity. *Journal of Clinical Endocrinology and Metabolism* 2008; 93: 771-778

Wisselink HW, Weusthuis RA, Eggink G, Hugenholtz J, Grobben GJ. Mannitol Production by Lactic Acid Bacteria: A Review. *International Dairy Journal* 2002; 12: 151-161

Wolever TMS, Piekarz A, Hollands M, Younker K. Sugar Alcohols and Diabetes: A Review. *Canadian Journal of Diabetes* 2002; 26: 356-362

Wood SG, John BA, Hawkins DR. The Pharmacokinetics and Metabolism of Sucralose in the Dog. *Food and Chemical Toxicology* 2000; 38: S99-S106

Yadav SK, Guleria P. Steviol Glycosides from Stevia: Biosynthesis Pathway Review and their Application in Foods and Medicine. *Critical Reviews in Food Science and Nutrition* 2012; 52: 988-998

Yallop KG, Sheppard SV, Smith DC. The Effect of Mannitol on Renal Function following Cardio-Pulmonary Bypass in Patients with Normal Pre-Operative Creatinine. *Journal of the Association of Anaesthetists of Great Britain and Ireland* 2008; 63: 576-582

Yang Q. Gain Weight by “Going Diet” Artificial Sweeteners and the Neurobiology of Sugar Cravings. *Yale Journal of Biology and Medicine* 2010; 83: 101-108

Yokozawa T, Kim HY, Cho EJ. Erythritol Attenuates the Diabetic Oxidative Stress through Modulating Glucose Metabolism and Lipid Peroxidation in Streptozotocin-Induced Diabetic Rats. *Journal of Agriculture and Food Chemistry* 2002; 50: 5485-5489

Zygler A, Wasik A, Kot-Wasik A, Namiesnik J. Determination of Nine High-Intensity Sweeteners in Various Foods by High-Performance Liquid Chromatography with Mass Spectrometric Detection. *Analytical and Bioanalytical Chemistry* 2011; 400: 2159-2172

