Studies on the Possible Mechanisms behind the Anti-diabetic Effects of Xylitol

By

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Submitted in fulfillment of the academic requirements for the degree of Master of Science in Biochemistry to the college of Agriculture, Engineering and Science at the University of KwaZulu-Natal, Durban, South Africa.

Supervisor: Dr MS Islam

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PREFACE

The experimental work described in this dissertation was carried out in the Department of Biochemistry, Under the School of Life Sciences, University of KwaZulu-Natal (Westville Campus), Durban, from February 2013 to November 2013, under the supervision of Dr Shahidul Islam.

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

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Chika Chukwuma

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Dr Shahidul Islam (Supervisor)

FACULTY OF SCIENCE AND AGRICULTURE

DECLARATION 1 - PLAGIARISM

I, **Chukwuma Chika Ifeanyi**, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
    a. Their words have been re-written but the general information attributed to them has been referenced
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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

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DECLARATION 2 - PUBLICATIONS

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

All work of the studies in the publications and presentation stated below was carried out by Chukwuma C.I. under the supervision of Islam M.S. (PhD), while the editorial work was done by Islam M.S. (PhD).

Publication 1

Chukwuma C.I. and Islam M.S. Studies on the possible mechanisms behind the anti-diabetic effects of xylitol. Department of Biochemistry, School of Life Sciences, College of Agriculture, Engineering and Sciences, University of KwaZulu-Natal, Durban, South Africa. **(in preparation)**

Publication 2

Chukwuma C.I. and Islam M.S. Xylitol improved in vivo anti-oxidative status in a normal and type 2 diabetes model of rats. Department of Biochemistry, School of Life Sciences, College of Agriculture, Engineering and Sciences, University of KwaZulu-Natal, Durban, South Africa. **(in preparation)**

PRESENTATIONS

Chukwuma C.I. and Islam M.S. Xylitol improved in vivo anti-oxidative status in a normal and type 2 diabetes model of rats. A poster presentation at the College of Agriculture, Engineering and Sciences Research Day, University of KwaZulu-Natal, Durban, South Africa on 1st November, 2013.

Signed:

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DEDICATION

THIS PROJECT WORK IS DEDICATED TO GOD ALMIGHTY, MY FATHER (LT. MR A. O. CHUKWUMA) AND MY DEAREST MOTHER (MRS B. CHUKWUMA)

ACKNOWLEDGEMENTS

First, I thank God Almighty for his grace, strength, knowledge, wisdom and guidance during the course of this study.

Second, I thank my supervisor, Dr Shahidul Islam, who was the pilot of this project.

Third, a vote of thanks goes to my family and my Guardian, Dr J.O. Chukwuma and family for their commitment to my well being and welfare, financially and morally

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I will not forget to appreciate my dearest friends, Obasi Amarachi and other fellow Nigerians in UKZN for their moral and relentless support.

LIST OF ABBREVIATIONS

ABBREVIATION	MEANING
Ace-K	Acesulfame potassium
ADIA	American Dietetic Association
ADA	American Diabetes Association
ADI	Accepted Daily Intake
AG	Alpha glucosidase
AM	Alpha amylase
AMPK	AMP-activated protein kinase
AND	Academy of Nutrition and Dietetics
ANSA	3 –amino-5-nitrosalicylic acid
ASs	Artificial sweeteners
B.W.	Body weight
BDA	British Diabetes Association
cAMP	Cyclic adenosine monophosphate
CHO	Carbohydrate
CVD	Cardiovascular diseases
DBA	Diabetic acarbose
DBC	Diabetic control
dH_2O	Distilled water

LIST OF ABBREVIATIONS CONTINUES

ABBREVIATION	MEANING
DNSA	3,5-dinitrosalicylic acid
DXYL	Diabetic xylitol
EDI	Estimated Daily Intake
FDA	Food and Drug Administration
FFA	Free fatty acid
FinDA	Finnish Diabetes Association
GAI	Glucose absorption index
GIIS	Glucose induced insulin secretion
GIP	Gastric inhibitory peptide
GIT	Gastrointestinal tract
GKS	Glucose Kreb's solution
GLUT-4	Glucose transporter type 4
GRAS	"Generally Regarded As Safe"
HFCS	High fructose corn syrup
HHS	Health and Human Services
HSD	Honestly significant difference
HSH	Hydrogenated starch hydrolysates
IDF	International Diabetes Federation
IFICF	International Food Information Council Foundation

LIST OF ABBREVIATIONS CONTINUES

ABBREVIATION	MEANING
ISs	Insulin secretagogues
M.W.	Molecular weight
NC	Normal control
NFBG	Non fasting blood glucose
NIEHS	National Institute for Environmental Health Science
NNSs	Non-nutritive sweeteners
NXYL	Normal xylitol
OCBS	O-methyl-benzenesulfonamide
OTZDs	oxathiazonedioxides
PKA	Protein kinase A
PKU	Phenylketonuria
PNPG	Paranitrophenyl-α-D-glucopyranoside
PPAR-γ	Peroxisome proliferator-activated receptor *gamma*
PR	Phenol red
reb	Rebaudioside
SAs	Sugar alcohols
SPSS	Statistical Package for the Social Sciences
SSs	Sugar substitutes
T2D	Type 2 diabetes

LIST OF ABBREVIATIONS CONTINUES

ABBREVIATION	MEANING
TZDs	Thiazolidines
WHO	World Health Organization

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ABSTRACT

Background and Objectives: Sugar substitutes have gained popularity in the management of diabetes, obesity and related diseases, due to their low calorie content compared to sucrose. Recent studies have reported that xylitol, a commonly used sugar alcohol has anti-diabetic properties. This study was therefore conducted to investigate the possible mechanism(s) behind the anti-diabetic effects of xylitol.

Materials and Methods: This study was carried out by investigating both *in vitro, ex vivo* and *in vivo* mechanisms. For *in vitro* studies, the inhibitory effects of different concentrations of xylitol (2.5, 5, 10, 20, 30 and 40%) on carbohydrate hydrolyzing and digestive enzymes; alpha-amylse and alpha-glucosidase were investigated. For the *ex vivo* studies, seven-week-old normal adult male Sprague-Dawley rats (B.W. 180-200 g) were sacrificed and the jejunum and psoas muscles were collected. The effect of increasing concentrations of xylitol (10, 20 and 40%) on intestinal glucose absorption was then examined in isolated rat jejunum. A 3 mM acarbose was used as positive control. Additionally, the effect of increasing concentrations of xylitol on muscle glucose uptake, with and without insulin (100 mU/mL) was investigated in the isolated rat psoas muscles, when 1mg/mL metformin was used as positive control. For the *in vivo* studies, adult Spagrue Dawley rats were randomly divided into five groups: normal control (NC), normal xylitol (NXYL), diabetic control (DBC), diabetic xylitol (DXYL) and diabetic acarbose (DBA). Type 2 diabetes was induced in the DBC, DXYL and DBA groups. After confirmation of diabetes, animals were fasted overnight for 16 hours, and a single bolus dose of either glucose (for NC and DBC groups) or glucose and xylitol (for NXYL and DXYL groups) or glucose and acarbose (for DBA group) solutions was orally administered to rats along side with 0.05% w/v phenol red as recovery marker. Animals were sacrificed exactly 1 hr after dose administration, and glucose absorption and related parameters were estimated in different segments of the gastrointestinal tract (GIT).

Results: Increasing concentrations of xylitol showed increasing inhibition of 4 U/mL alpha-amylase (IC_{50} = 21.69 xylitol) and 1 U/mL alpha-glucosidase (IC_{50} = 17.58 xylitol) activities with the highest inhibition at 30% and 40%. Also increasing concentrations of xylitol showed lower glucose intestinal absorption, which was significantly lower at 40% Xylitol compared to

the control, 10 and 20% xylitol, but only relatively lower compared to 3mM acarbose. Furthermore, increasing concentrations of xylitol showed increasing glucose muscle uptake, which was significantly higher at 40% Xylitol compared to the control, but only relatively higher compared to 10, 20% xylitol and 1 mg/mL metformin. The presence of insulin did not significantly affect muscle glucose uptake of xylitol. Additionally, oral single bolus dose of xylitol inhibited small intestinal glucose absorption, delayed gastric emptying and accelerated digesta transit in the GIT compared to the respective controls. Acarbose did not significantly affect intestinal glucose absorption in both *in vivo* and *in vitro* conditions.

Discussion and conclusion: The anti-diabetic effects of xylitol may not only be due to the slower gastric emptying and lower carbohydrate digestion and glucose absorption from the small intestinal mucosa. It may also be due to improving insulin action or exhibiting an insulin-like effect on skeletal muscle, thus increasing muscle glucose uptake and utilization, which may also be mimicked under *in vivo* conditions.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Diabetes is a major threat to global public health that is imposing socio-economic burden around the world especially in the developing countries. The prevalence of this disease is increasing at an alarming ratedue to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity (Wild *etal*., 2004). About 171 million peoplewere with diabetes in the year 2000 whichhas been projected to become about 336 million people in the year 2030 (Wild *et al*., 2004). Supporting this projection, this figure was already more than 371 million people in 2012 and more than 471 billion USD was spent on health care expenditure for diabetes in the same year (IDF, 2012).

TABLE 1.1: Global estimate and prevalence of people with diabetes (20-79 years) in 2012 and 2030 (IDF Diabetes atlas, 5th edition, 2012 updates).

	2012	**2030**
Number of people from ages 20-79 years with diabetes (million)	371	551.8
Comparative diabetes prevalence (%)	8.3	8.9

Furthermore,the mortality rate of diabetes is also increasing concomitantly with its prevalence. According to the International Diabetes Federation (2012),about 4.8 million people died in 2012 due to diabetes, which was approximately 8.6% of all-cause of mortality and about 4.2% higher than 2011. There is therefore a great need for more research to discover better and more strategic approach towards combating diabetes.

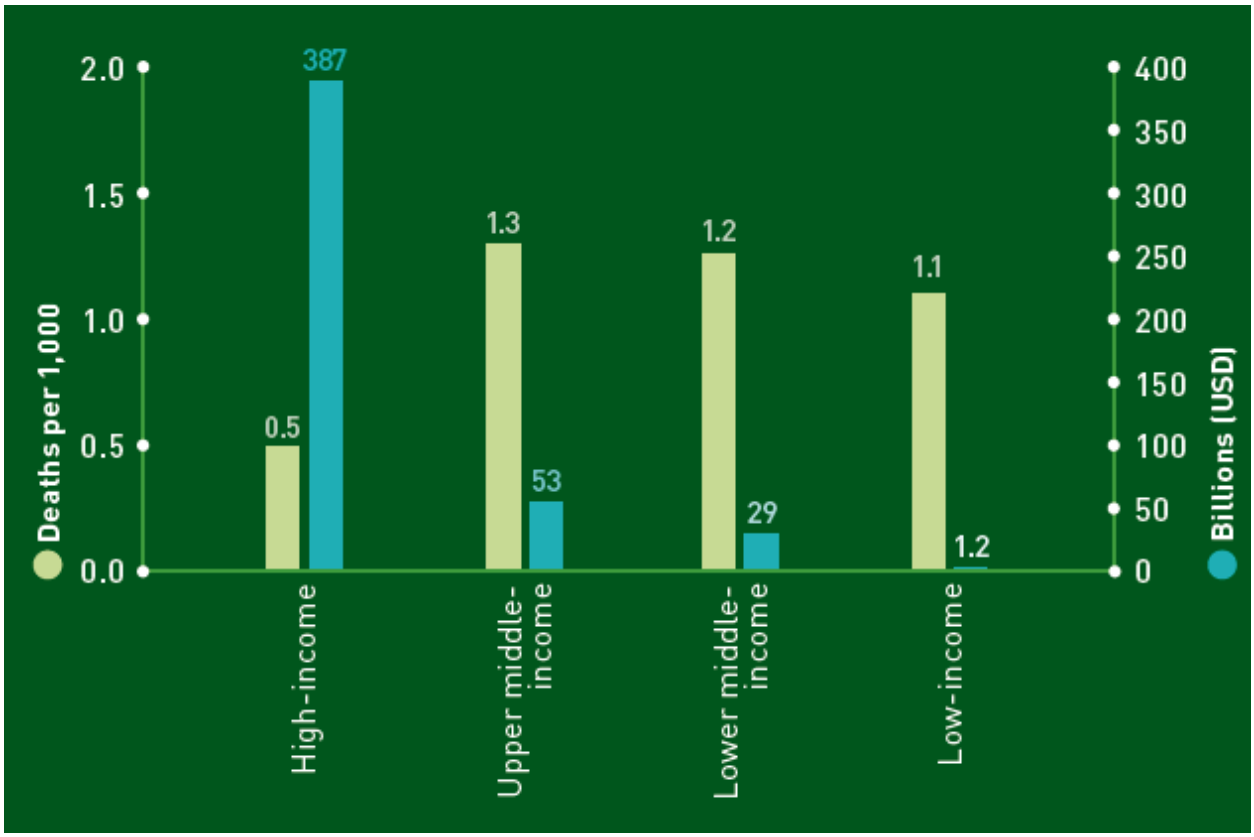


FIGURE 1.1: Global healthcare expenditures and deaths per 1,000 due to diabetes by income group in 2012 (IDF Diabetes atlas, 5th edition, 2012 updates).

Like other parts of the world, diabetes has also become a serious issue in Africa. In South Africa for example, IDF estimated that approximately 1.9 million of 30 million adults between 20 to 79 years are with diabetes (Amod*etal*., 2012). It is quite unfortunate that diabetes and its complications are increasing at an alarming rate, especially among the people in black and Indian community, and about 50-85% of diabetes sufferers (especially in rural areas) remain undiagnosed (Amod*et al*., 2012).

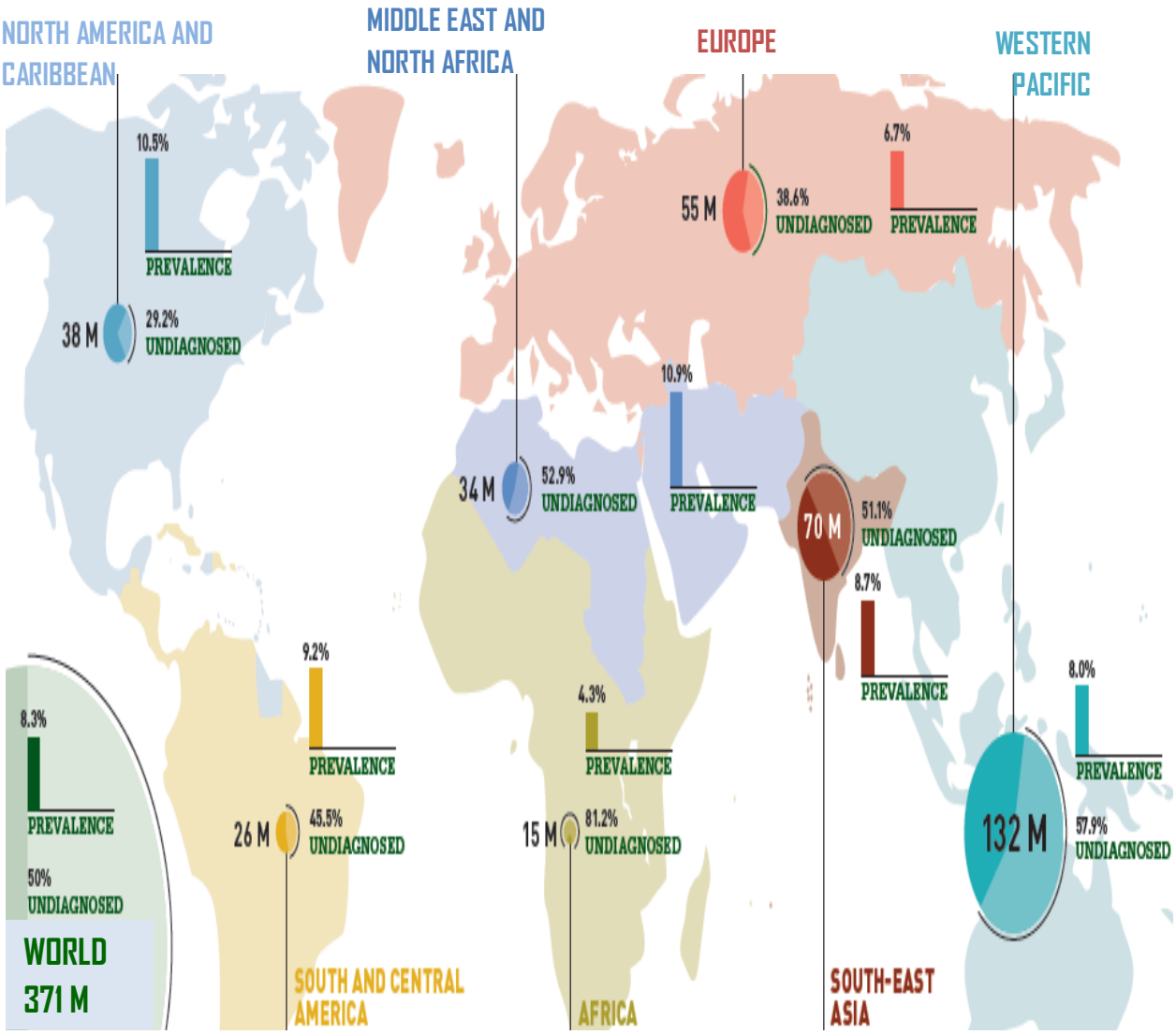


FIGURE 1.2: Global map showing regional estimates and prevalence of people with diabetes (20-79 years) in 2012 (IDF Diabetes atlas, 5th edition, 2012 updates).

Among the types of diabetes, type 2 diabetes (T2D) is known to have the highest prevalence, which is between 90% - 95% of all diabetes cases (Loghmani, 2005; Sheri *et al*., 2010). This research is therefore focused on T2D, because of its higher prevalence compared to other types and the dietary related factors associated with it, having in mind today's poor and unhealthy eating style.

Artificial or synthetic sweeteners such as saccharin, acesulfame potassium (Ace-K), sucralose, neotame and aspartame have been known to be sugar substitutes especially for diabetic individuals because of their significantly higher sweetness than sucrose with very low or no calorific values (Islam and Indarjit, 2012). Nevertheless, the short or long term chronic consumption of these sweeteners is associated with a number of adverse effects on health(Abdelaziz and Ashour, 2011; Bell *et al.,* 2002; Clayson, 1984; Kroger *et al.,* 2006), which has been a major concern to consumers. For these reasons natural sweeteners like honey, high fructose corn syrup, fructose, maple syrup, molasses etc. are usually used as alternative source of sweeteners. However, it has been reported that the chronic or over consumption of these sweeteners may cause severe health problems such as overweight, obesity, type 2 diabetes (T2D) and many other diseases related to metabolic syndrome (Stanhope, 2011). On the other hand, sugar alcohols are preferred alternatives to other natural and artificial sweeteners, and more attention has been given to xylitol because of its better effects on health compared to other sugar alcohols and, lower caloric value (2.4 kcal vs 4.0 kcal/g), insulinemic response, lower glycemic index (13) and similar sweetness compared to sucrose (Islam, 2011).

Additionally, xylitol has several unique beneficial effects such as dental protection (Spliet*h et al*., 2009; Twetman, 2001) and control and prevention of obesity, diabetes and related metabolic disorders (Amo*et al*., 2011; Islam, 2011; Islam and Indarjit, 2012; Kishore *et al*., 2012). Xylitol is used in common products like table top sweeteners, candies, tooth pastes and anti-microbial mouth wash.

1.2 Aim and Objectives

In a recent study, it was reported that xylitol can be a better sweetener than sucrose to maintain diabetes-related parameters at physiologically safer and stable conditions (Islam, 2011).In another study, the *in vivo* anti-diabetic effects of xylitol were reported (Islam and Indarjit, 2012). In these studies, xylitol significantly reduced the NFBG and also improved most diabetes-related parameters in normal and T2D model of rats. Furthermore, it has been reported that xylitol consumption significantly prolongs gastric emptying, decreases food intake and accelerates intestinal transit of nutrients compared to glucose, fructose and some other polyols in normal human subjects and experimental animals (Salminen*etal*., 1984 and 1989; Shafer *etal*., 1987). This was demonstrated in a recent study (Islam and Indarjit, 2012), where lower food and fluid intake was reported in rats fed with 10% xylitol solution compared to the diabetic control. In this study, it was predicted that faster intestinal transit and slower gastric emptyingmight reduce the rate of intestinal nutrient absorption. It could be possible that xylitol may have delayed intestinal absorption of glucose or prevented the digestion of carbohydrate by digestive enzymes such as alpha glucosidase and alpha amylase, thus lowering NFBG and reducing food intake compared to diabetic control rats. Furthermore, as mentioned above, insulin plays a significant role in the cell uptake of circulating glucose. We speculate that the insulinotropic effect of xylitol, as reported by Islam and Indarjit (2012) might be involved in improving circulating glucose uptake, especially in muscle and fat cells. However, these possiblemechanism(s) behind the anti-diabetic effects of xylitol have not been studied, especially under diabetic condition. The present study was therefore aimed at investigating the possible mechanism(s)behind the anti-diabetic effects of xylitol in normal and experimentally-induced T2D model of rats.

CHAPTER 2

LITERATURE REVIEW

2.1 Background

T2D is identified by hyperglycemia, insulin resistance and relative insulin deficiency (Olokoba*et al*., 2012). The classic symptoms of T2D are excess thirst, frequent urination and constant hunger. In progressive cases T2D can cause complication like cardiovascular diseases (CVD), kidney diseases, nerve diseases and blindness, which is a significant cause of mortality and morbidity (Colberg*et al*., 2010).

During T2D,there exist inadequate response of myocytes and adipocytes to insulin and subsequent insufficient insulin production to compensate for this defect (Colberg*et al*., 2010). Inadequate insulin production from beta cells, also known as impaired glucose induced insulin secretion is caused by progressive beta-cell dysfunctions, which contributes to persistent hyperglycemia.Some genetic and environmental factors are also involved in the development of T2D. The role of genetic implications has not been clearly defined (ADA, 2010). Environmental factors include dietary factors and life style. For example, high consumption of simple carbohydrates and saturated fats combined with inadequate exercise can result in obesity followed by insulin resistance and T2D. The risk of T2D is believed to increase with obesity, hypertension or dyslipidemia, physical inactivity and age (Colberg*et al*., 2010; Warjeet, 2011).

2.2 Carbohydrate Metabolism and Blood Glucose Regulation

Carbohydrate (CHO) from starch is a major human dietary source of glucose, a product of hydrolysis. This is absorbed in the small intestine, influencing postprandial blood glucose levels. Thusthe rate and extent of CHO digestion and glucose absorption is linked to glycemia-related problems like diabetes, obesity and related metabolic disorders (Dhital*et al*., 2013). CHO is broken down or hydrolyzed into smaller oligosaccharides, disaccharides and monosaccharides by important CHO hydrolyzing enzymes, alpha amylase (AM) and alpha glucosidase (AG).

Alpha amylases are a group of calcium dependent enzymes that catalyzes the random break down of amylose and amylopectin units in starch to yield smaller CHO units like maltotriose, maltose and glucose (Qian*et al*., 1997). They are known to hydrolyze the alpha bonds of alpha-linked polysaccharides. In human, they are found in several tissues, but are mostly present in the

saliva and pancreatic juice, which both have similar amino acid sequences (Pasero*et al*., 1986). Salivary AM is most effective at an optimum pH between 6.7 to7.0. It is usually inactivated in the stomach by the acidic pH of gastric acid, but studies have shown that both starch and products from starch hydrolysis are able to protect salivary AM from gastric acid inactivation (Rosenblum*et al*.,1988).

Alpha glucosidases are a group of enzymes that belong to the family of the glycoside hydrolases, which catalyzes the release of alpha glucose from the reducing terminal end of alpha glycosyl residue-possessing substrates (Okuyama*et al*., 2005). In human, they are present in the intestinal mucosa as two membrane bounded protein complexes (maltase-glucoamylase and sucrase-isomaltase), which catalyzes from the reducing, end the hydrolysis of α-1,4-glycosidic linkages resulting from AM starch degradation to form free glucose (Dhital*et al*., 2013).

Glucose resulting from the digestion of starch and other carbohydrates is absorbed into the blood via the wall of the duodenum. The rate of gastric emptying and intestinal transit of nutrient is an important factor in gastrointestinal nutrient digestion and absorption (Islam and Indarjit, 2012). Nutrient gastric emptying is the movement of ingested food down into the small intestine from the stomach, while nutrient intestinal transit time is the time it takes ingested food to travel through the entire length of the intestine. It is believed that faster intestinal transit and delayed gastric emptying of nutrient may be the cause of slow intestinal nutrient absorption and reduced food intake (Salminen*et al*., 1984; Shafer *et al*., 1987).

Furthermore, the rate and manner of gastric emptying and intestinal transit of ingested food partly depends on the nature and concentration of what was ingested (Elias *et al*., 1968; Moran and McHugh, 1981). For example, fructose is emptied from the stomach more quickly than glucose, while some polyols like xylitol, sorbitol and lactitol have shorter intestinal transit time than glucose, which is believed to be a major contributor to abdominal pain and diarrhea normally experienced by normal individuals after ingesting moderate to large amounts of fructose and the above-mentioned polyols (Salminen*et al*., 1989).

Unlike fructose and polyols, these abdominal discomforts do not occur after ingestion of equivalent or comparable amounts of glucose. Studies have shown that aqueous glucose solution has an exponential gastric emptying rate that is inversely proportional to its concentration (Elias

et al., 1968). In fact, unlike fructose, ingestion of glucose stimulates gastric inhibitory peptide (GIP) secretion (Sykes *et al*., 1980), which has been reported to play a major role in the proposed mediation of delayed gastric emptying by enterogastrone hormone (Brown, 1982). Despite the slow gastric emptying rate of glucose solution, glucoseis not rapidly transported along the intestine. In a study conducted by Salminen*et al*.,(1989) on normal subjects to examine the gastrointestinal transit rate of glucose compared to xylitol, glucose travelled through the intestine at a much slower rate, which was attributed to its rate and manner of absorption.

Once glucose is absorbed, it immediately moves into the blood and circulation. The body therefore possesses appropriate mechanisms to ensure physiological blood glucose homeostasis (Figure 2.1). Glucose is removed from the blood when not in use by converting excess blood glucose to glycogen which is stored in the liver. When the body requires energy for normal physiological functions, and when there is a fall in blood glucose level, stored glycogen is broken down into glucose, which is then released into the blood (Mathews *et al*., 2000). Glucagon and insulin are the two major hormones in the body that work consistently to regulate and maintain physiological blood glucose homeostasis.

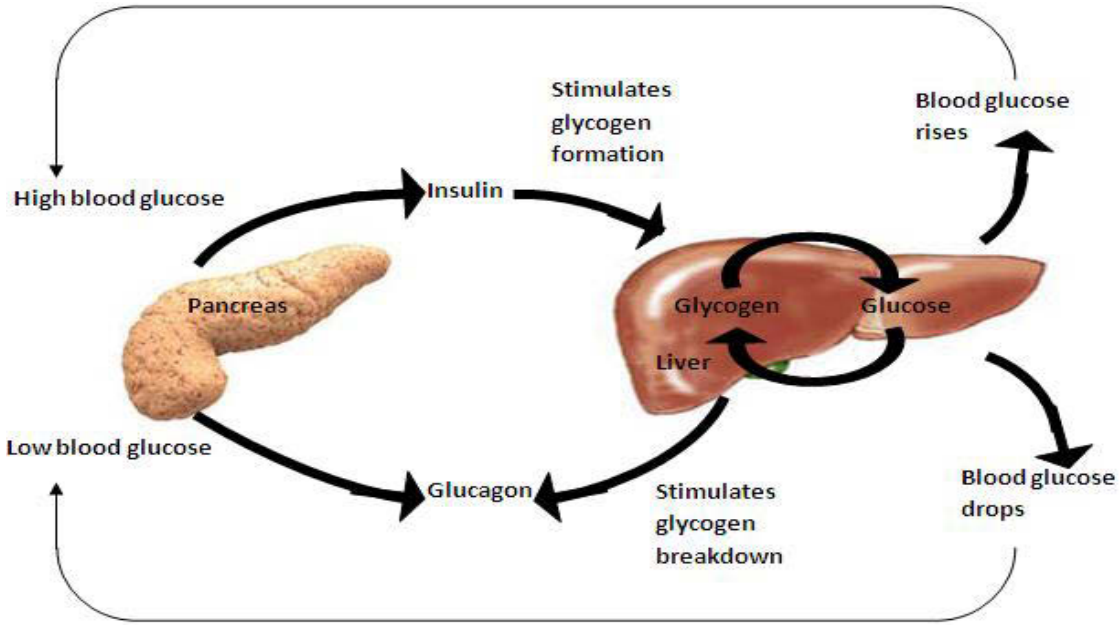


FIGURE 2.1: Hormonal regulation of blood glucose level (Mathews *et al*., 2000).

Glucagon is produced by the alpha cells of the pancreas when there is low blood glucose. Glucagon hormone causes the cAMP dependent activation of glycogen phosphorylase, which then catalyzes the phosphorylytic breakdown of glycogen to release glucose-6-phosphate into the blood(Jiang and Zhang, 2003). Glucagon hormone also promotes gluconeogenesis when glycogen reserve is very minimal. When blood glucose rises, either due to glycogen break down or intestinal glucose absorption, the beta-cells of the pancreas releases insulin which promotes cell glucose uptake via cell membrane glucose transporter type 4 (GLUT 4); glycolysis; inhibition of glucagon secretion by alpha cells; and also signals the activation of glycogen synthase that catalyzes the conversion of glucose to glycogen, thus normalizing blood glucose level (Mathews *et al*., 2000). Insulin resistance is a major disorder in glucose metabolism and homeostasis that can contribute to hyperglycemic conditions in T2D.

2.3 Insulin Resistance

Insulin resistance is involved in the progression of T2D. For example, during the onset of T2D, there may exist normal blood glucose condition, while the individual gradually develops insulin resistance and subsequent inadequate blood glucose uptake. If this remains undiagnosed and untreated, it may contribute to persistent hyperglycemia in T2D. Insulin resistance has been found to be associated with a number of factors, which include genetic factors and environmental factors. However, environmental factors such as inactivity or lack of exercise, aging, type of diet and obesity are still the most influencing factors that can contribute to the development of insulin resistance (Kahn, 2003).

Insulin resistance occurs when affected cells such as myocytes,adipocytes and hepatocytes do not effectively respond to insulin hormone signaling. Cells become insensitive to insulin and thus the release of insulin hormone into the blood does not effectively normalize blood glucose level. However, the effect of insulin resistance can vary from one organ or tissue to another. For example, in the liver, it can result in reduced glycogenesis and glycogen storage and increased glycogenolysis and release of glucose into circulation, while muscle and fat cells donot adequately take up circulating glucose during insulin resistance (Mathews *et al*., 2000).

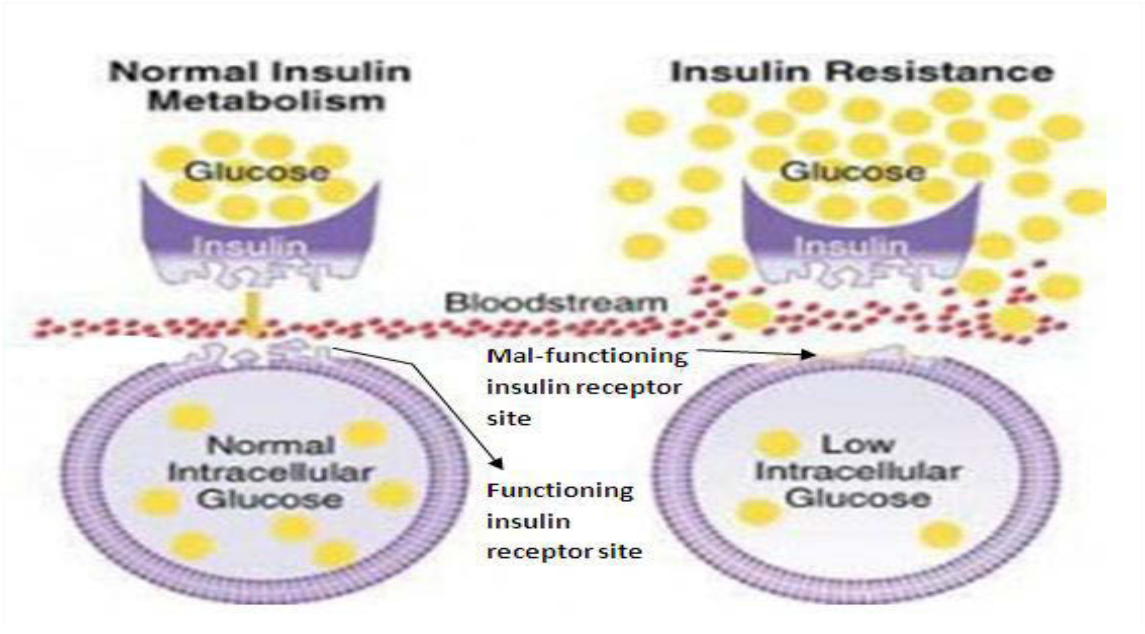


FIGURE 2.2: Cells showing insulin resistance and insulin sensitivity (www.encyclopedia.com).

Apart from blood glucose regulation and homeostasis, insulin is also important in the regulation of body fat metabolism. It is critical for promoting uptake of circulating free fatty acid (FFA); differentiation and maturation of adipocytes; lipogenesis; and inhibition of lipolysis (Kahn and Flier, 2000). Therefore, a defect in fat metabolism, subsequent abnormally high plasma FFA and triacylglyceride and abnormally low plasma high density lipoprotein levels can occur during insulin resistance, which can predispose an insulin resistant patient to obesity, metabolic syndrome and other diabetes related complications (Haag and Dippenaar, 2005; Rabol*et al*., 2011).

In order to compensate for insulin resistance and insensitivity of cells, more insulin is being released into the blood. This will eventually cause hyperinsulinemia, a condition where there is an abnormally high plasma insulin level. Hyperinsulinemia will signal for more energy storage into adipocytes, new fatty tissues formation and hunger, thus promoting weight gain, a major feature of metabolic syndrome (Isganaitis and Lustig, 2005).

2.4 Pancreatic Beta cell Dysfunction and Impaired Insulin Secretion in T2D

The pancreas is the sole organ that produces insulin in the body. It is predominantly dominated with beta cells among several types of secretory cells,which collectively make up the islet of langerhans within the pancreas. Insulin hormone is secreted by these endocrine secretory beta-cells, and progressive beta-cell failure is associated with the development of type 1 and T2D. Beta-cell failure is caused by auto-immune related beta-cell damage in type 1 diabetes, while insulin resistance related factors play a major role in the development of beta-cell failure in T2D, which is manifested by failure of glucose to stimulate insulin secretion by beta-cells (Cnop*et al*., 2005; Ogawa *et al*., 1992).

As previously stated, insulin resistance can cause hyperinsulinemia, when the body tries to compensate for insulin insensitivity of cells by producing more insulin. This abnormally high demand for production and secretion may eventually exhaust the beta-cells, thus leading to impaired insulin secretion and glucose intolerance, which in the presence of persistent hyperglycemia forms the major characteristics ofT2D (Cnop*et al*., 2005; Li *et al*., 2011).

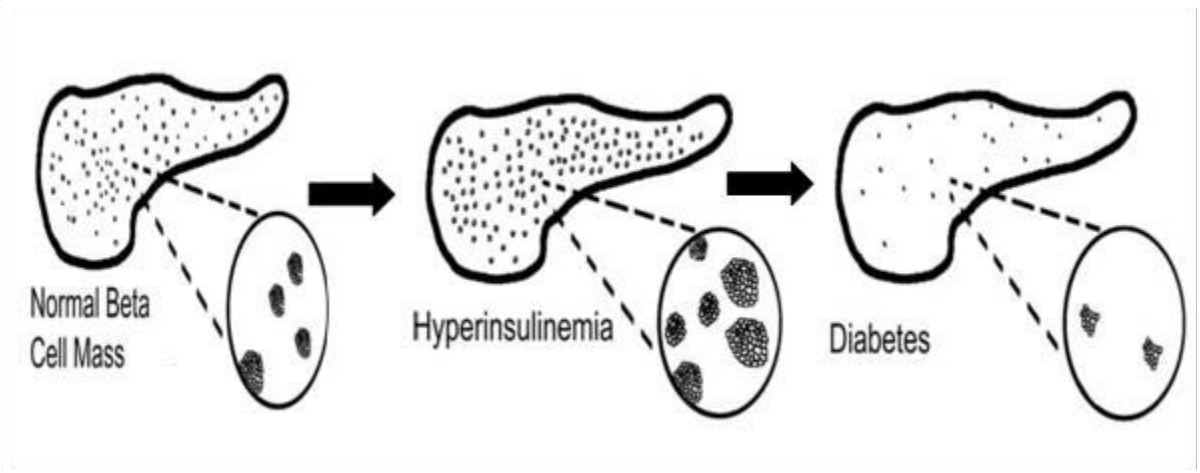


FIGURE 2.3: Schematic diagram showing the changes in beta-cell mass accompanying progression of T2D (Souza *et al*., 2006).

In a previous study, Ogawa *et al*.,(1992) were able to provide some evidences supporting the correlation between insulin resistance, beta-cell dysfunction and impaired insulin secretion as major characteristics of T2D.They observed that the islet mass and other indices of insulin

resistance in insulin resistant diabetic rats were lesser compared to insulin resistant non-diabetic rats, indicating inadequate compensation by beta-cells for insulin resistance and impaired insulin secretion in the diabetic rats. From these observations, they reported that insulin resistance alone was not sufficient to cause T2D in the diabetic rats, suggesting the presence of a concomitant beta-cell disorder whenever diabetic hyperglycemia due to insulin resistance occurred. Supporting this, Cnop*et* al., 2005 hypothetically reported that a disorder in glucose-induced insulin secretion (GIIS) during the development of T2D in human may be as a result of a derangement in beta-cell function and mass.

Furthermore, under hyperglycemic conditions, glucotoxicity may occur, which may adversely affect the mass, function and survival of beta-cells. This is believed to be a majorcontributor to GIIS in T2D (Cnop*et al*., 2005). Although, the mechanism behind hyperglycemic glucotoxicity on beta-cells still remains a debate, several studies have reported reduced expression of pancreatic GLUT 2 and glucokinase genes and subsequent reduced GIIS in diabetic insulin resistant rats (Cnop*et al*., 2005; Ogawa *et al*., 1992).

Several therapeutic approaches have been used to ameliorate diabetes. These approaches range from synthetic drugs and medicinal plants to dieting and exercise. For T2D, treatment is achieved by controlling the intake of calorie-rich and postprandial blood glucose-increasing diet and use of hypoglycemic drugs (Patel*et al*., 2012). However some therapeutic agents are also aimed at preventing and treating diabetes related complications and metabolic syndrome.

2.5 Therapeutic Agents for Treatment of T2D and Their Modes of Action

There are several anti-hyperglycemicand glucose lowering agents that are used in the treatment of T2D. They act differentlyto maintain lowblood glucose. Most of them are orally administered, except for insulin and insulin preparations which are administered parenterally (Warjeet, 2011). Most of these anti-diabetic drugs have been tried on experimental models like rodents, in which T2D has been induced by pharmacological, surgical or genetic manipulations to examine anti-diabetic effects (Fröde and Medeiros, 2008). Depending on their mechanism of action, anti-diabetic drugs or agents used in the treatment of T2D are classified into several groups such as:

insulin and insulin secretagogues, insulin sensitizers, suppressor of hepatic glucose production, alpha glucosidase inhibitors and so on.

2.5.1 Insulin and Insulin Secretagogues

Most type 2 diabetic patients may eventually develop progressive pancreatic beta-cell failure and impaired insulin secretion, and as such may need insulin to control hyperglycemia (Hamaty, 2011). Insulin as a drug is an injectableanalogue of the normal physiological insulin with similar function of promoting cell uptake of glucose, glucose disposal and storage of glucose as glycogen, thus lowering blood glucose level. Therapeutic insulin is divided into two types depending on the mode of action: long-acting (basal) insulin, which is usually taken at bed time to maintain normal blood glucose and rapid acting (prandial or bolus) insulin which is usually injected after a meal to control postprandial blood glucose rise (Hamaty, 2011).

Insulin secretagogues(ISs) are T2D medications that are used to remedy impaired insulin secretion in type 2 diabetic patients. They help the pancreas to produce and secret insulin for blood glucose homeostasis (Patel *et al*., 2012). Some existing commonly used insulin secretagogues are the sulphonylureas like glyburide, gliclazide and glipizide, which are sometimes used in combination with other hypoglycemic agents. However, it has been reported that this class of ISs are associated with hyperinsulinemia; risk of hypoglycemia; inadequate glycemic control (in type 2 diabetes patients); gradual failure in beta-cell function; and weight gain, while meglitinides like repaglinde, which is another class of ISs and a prandial glucose regulator is believed to have better glycemic control and lower risk of hypoglycemia than sulphonylureas (Davies, 2002).

2.5.2 Insulin Sensitizers

Insulin resistance and insensitivity in the body tissue, especially in the muscles are major metabolic defects associated withT2D.Insulin produced cannot adequately induce blood glucose uptake or disposal in these tissues, which contributesto hyperglycemia. Insulin sensitivity improvement drugs, such as thiazolidines (TZDs)and biguanides like metforminwork by

improving the insulin insensitivity and insulin dependent glucose uptake in muscles and other tissues, hence improving metabolic control in T2D patients (Hauner, 2002; Klip and Leiter, 1990; Sirtori and Pasik, 1994). Although, the mechanism or mode of action of TZDs as anti-diabetic agents at molecular level still remains unclear, it has been reported that TDZs can reduce serum glucose, insulin and triglyceride level and also increase blood glucose uptake when used to treat type 2 diabetic patients (Kahn *et al*.,2000). Hauner (2002) reported that the mechanism of action of TDZs involves a Peroxisome proliferator-activated receptor *gamma* (PPAR-γ)-dependent transcription control of genes involved in glucose and fat metabolism in adipose and muscle tissues, which are promoted via an endocrine signal from adipocytes.

Common anti-diabetic drugs belonging to this class include bigaunides (e.g. metformin), troglitazone, rosiglitazone and pioglitazone. Some have been hypothesized to have potential therapy against T2D related complications such ascardiovascular diseases (Charbonnel*et al*., 2004; Hauner, 2002). Despite this, some TDZs like rosiglitazone is believed to be associated with some adverse effects like risk of weight gain, coronary heart disease, heart attack and some other vascular disease (Hussein *et al*., 2004).

Metformin treatment has been reported to be associated with vitamin B_{12}deficiency and increased risk of lactic acidosis especially in individuals with renal or CVD (Bailey and Turner, 1996; liu*et al*., 2006). However it has been reported to be associated with reduced risk of cardiovascular diseases (CVD)and all-cause mortality in type 2diabetic patients with obesity and some beneficial effects compared to some anti-diabetic drugs such as sulphonylureas, insulin etc (Ekstrom *et al*., 2012; Kahn *et al*., 2006; Kooy*et al*., 2009). Studies conducted by Ekström*et al*.,(2012) showed lower risk of CVD and acidosis or serious infection in metformin treated type 2 diabetic patients than some oral glycemic drugs and insulin, concluding that the beneficial effects of metformin outweighs the risk of adverse effects.

2.5.3 Suppressors of Hepatic Glucose Production

Hepatic glycogenolysis and gluconeogenesis are two glucagon-dependent glucose production processes that serves as major sources of glucose for body metabolic functions, during fasting and when blood glucose drops. The inability of insulin to suppress these processes and the

hormonal and enzymatic factors that promotes them is a major etiological factor in the development of hyperglycemia in type 2 diabetic patients (DeFronzo*et al*., 1982; Postic*et al*., 2004). These processes, which are cyclic AMP (cAMP) dependent,are believed to be major targets for some classes of anti-diabetic drugs (Miller*et al*., 2013).Glucagon binding to cell membrane receptors causes the activation of adenylatecyclase, and subsequent cAMP production, which activates protein kinase A (PKA). Activated PKA phosphorylates and activates target proteins that signals or up regulates hepatic glucose output (Jiang and Zhang, 2005).

Anti-diabetic drug belonging to this class act by down regulating glycogenolysis and gluconeogenesis, which are major processes that increase hepatic glucose output.Thus, reducing the amount of glucose going into circulation when there is high blood glucose level. Although the molecular mechanism behind the anti-diabetic action of biguanides have been previously suggested to be via the enhancement of glucose disposal and activation of the enzyme AMP-activated protein kinase (AMPK), a cellular glucose uptake inducer (Goodazi and Bryer-Ash, 2005; Inzucchi*et al*., 1998; Zhou *etal*., 2001),recent studies have reported biguanides to be a suppressor of hepatic glucose production (Viollet *et al*., 2012), and metformin an antagonist of glucagon action (Miller *et al*., 2013).According to Miller *et al*., 2013, metformin treated mouse exhibited accumulation of AMP (an adenylatecyclase inhibitor) in the liver cells and increased PKA activity and target protein phosphorylation, indicatingsuppression or blocking of glucagon-dependent hepatic glucose output and hypoglycemic function.

2.5.4 Alpha Glucosidase Inhibitors

This class of oral glycemic drug work by preventing postprandial hyperglycemia (Reuser and Wisselaar, 1994). They delay the digestion of carbohydrate like starch and sucrose, and also delay the subsequent absorption of absorbable monosaccharides like glucose resulting from carbohydrate digestion (Bisschoff, 1994; Patel *et al*., 2012). It is believed that alpha glucosidase inhibitors are saccharides that competitively inhibit carbohydrate hydrolyzing enzymes like alpha glucosidase and alpha amylase (Bischoff, 1995; Horii *et al*., 1986; kim*et al*., 1999).

Studies have shown that acarbose, a common alpha glucosidase inhibitor used in the treatment of T2D, improved metabolic state and also reduced blood glucose levels in diabetic animals, which subsequently reduced non-enzymatic glycation of protein and haemoglobin. These effects and further beneficial effects of acarbose against nephropathy, neuropathy, retinopathy, endothelial dysfunctions and CVD problems make it a potential therapy for prevention of T2D complications (Bischoff, 1995; Standl and Schnell, 2012).

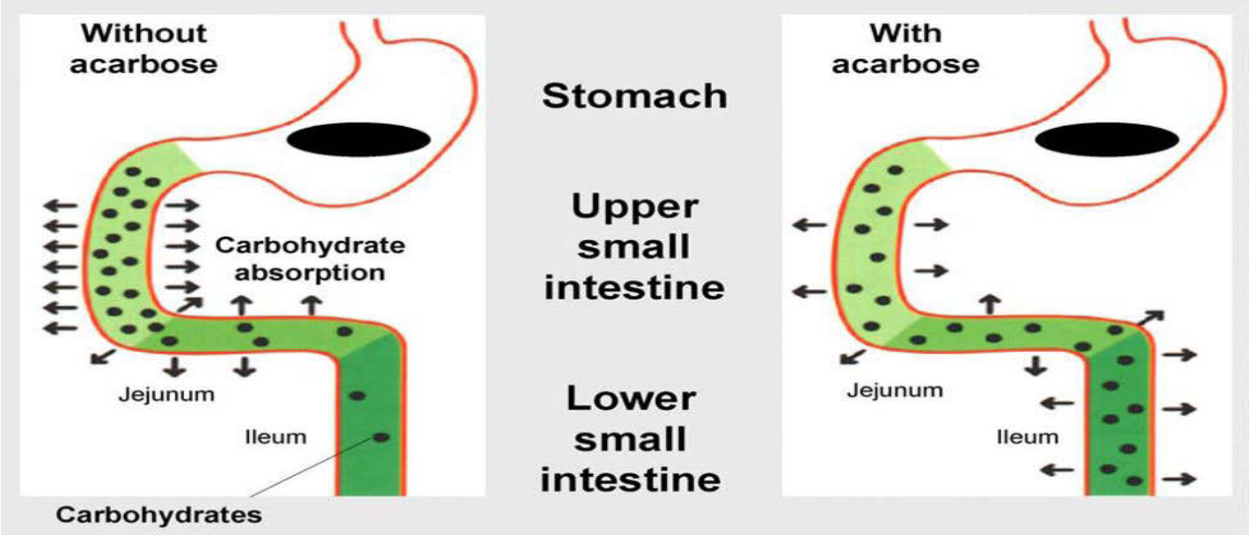


FIGURE 2.4: Non-systemic action of acarbose to delay carbohydrate absorption (Standl and Schnell, 2012).

Furthermore, the effects of alpha glucosidase inhibitors on nutrient gastric emptying rate and gastrointestinal transit time have also been investigated in both diabetic and normal subjects. Kawaqishi*et al*.,(1997) and Hücking*et al*.,(2005) reported that acarbose and voglibose did not delay or alter the rate of gastric emptying in non-insulin dependent diabetic patients. However, in a study conducted by Ladas *et al*.,(1992) to investigate the effects of alpha glucosidase inhibitors on nutrient gastrointestinal transit time in normal subjects it was reported that 100mg of acarbose increased the rate of nutrient mouth to cecum transit time and the amount of carbohydrate reaching the colon by inducing mal-absorption of carbohydrate. Reduced absorption of nutrient

in a diet can facilitate movement of unabsorbed nutrient down the gastrointestinal tract and towards the large intestine, which can cause osmotic diarrhea in the colon.

Nevertheless, the risk of development of adverse effects with the use of various hypoglycemic drugs is not an exception for alpha glucosidase inhibitors. It has been reported that treatment with acarbose was associated with abnormal increase in serum transaminase levels, which is an indicator for hepatotoxic effect of this drug; hence it is contra-indicated for patients with cirrhosis (Carlson, 2000). Oral hypoglycemic drugs are usually of synthetic origin and relatively expensive, and because of these and other adverse effects associated with these conventional chemically originated drugs, other therapeutic agents like some sugar substitutes have gained popularity in the management of diabetes, obesity and related diseases without sacrificing sweetness.

2.6 Sugar Substitutes and Artificial Sweeteners

Regular table sugar is normally used as a sweetener by most individuals and also in many consumables that contains mainly sucrose. Sucrose is a disaccharide that is rapidly digested and broken down into glucose and fructose by sucrase at the outer brush-border of the small intestine. It is contained in some fruits and vegetables, which however contributes relatively lesser amounts compared to that derived from table sugar and sucrose added to manufactured food (Southgate *et al*., 1978). Fructose and glucose resulting from sucrose digestionare equimolarin amount, although about 20% of the fructose may become converted to glucose before absorption (Brunzell, 1978; Nutall and Cannon, 1981). Glucose is rapidly absorbed via a mechanism of facilitated diffusion. For this reason, large consumption of sucrose has a direct and quick impact on blood glucose and subsequently on other metabolic parameters, which could be very detrimental for diabetic patients (Olefsky and Crapo, 1980). Furthermore, several studies have reported that detrimental positive balance in calorie and body weight, obesity as well as uncontrolled loss of body weight can be caused by over consumption of sucrose and other refined sugars (Harrington, 2008; Malik *et al*., 2006).

Despite the detrimental effects associated with sugar consumption, the consumption and demand for sugar is still increasing. Supporting this, it was reported that between 2006 and 2008, the

global demand of sugar increased by about 3.5million tons (Deshpande and Jadad, 2008). The increasing mortality and morbidity rate associated with T2D and related diseases over the years have been a case of concern. In the 1980's, both the Finnish Diabetes Association (FinDA) and the British Diabetes Association (BDA) recommended the continued restriction of sucrose and exclusion of rapidly absorbed mono- and disaccharides, except during emergency cases like hypoglycemia (BDA, 1982 and Huttunen *et al*., 1982). Howevre, the consumption of a modest amount of sucrose was regarded as acceptable, but may affect metabolic control (BDA, 1984).

Artificial sweeteners (ASs) and sugar substitutes (SSs) have now become a replacement for regular table sugar or sucrose in the diet of most people, especially in diabetic and obese individuals. They have found increasing application in a wide range of products like table top sweeteners, baked products, syrups, candies, sweet toppings, beverages, pharmaceuticals, sugar free desserts etc, because of their beneficial effects like relatively higher or similar sweetness levels with little or negligible effect on postprandial glucose, serum insulin and caloric value compared to sucrose (Islam, 2011; Islam and Indarjit, 2012).

Sugar substitutes, are substances that can be used to replace regular table sugar while giving about similar sweetness, and ASs are believed to be a group or class of SSs that are non-nutritive intense sweeteners of synthetic origin, although a few are derived from natural sources (Mayo clinic, 2010). Natural or nutritive sweeteners on the other hand are a second class of SSs that are majorly derived from natural source and have approximately same sweetness power as sucrose. Nevertheless, SSs are sometimes grouped into subclasses according to their calorie content: nutritive sweeteners provide energy with sweetness, while non-nutritive sweeteners are also sweet but do not provide energy (ADIA, 1998)

2.6.1 Synthetic and Non-nutritive Sweeteners

These groups of SSs are also called ASs, which are basically of synthetic origin. They are intense sweeteners with sweetness power ranging from 30 to 13,000 times sweeter than sucrose (Mayhew *et al*., 2003). They are known to offer consumers the taste of sweetness with little or no

calorie, and as such can help in weight gain and blood glucose control (ADIA, 1998). Furthermore, they are quite compatible with other food ingredients and relatively stable under different environments and conditions like heat. Their synergistic ability has allowed their combined use with other sweeteners to give a better blend of sweetness and sweetness powercompared to individual taste, and as such only a relatively very small amount of ASs are required to improve overall sweetness and taste (ADIA, 1998). These qualities have increased the popularity and use of ASs.

Six ASs have been reported to be commonly used by overweight, obese and diabetic individuals, which include saccharin, aspartame, neotame, sucralose, acesulfame potassium (Ace-K) and stevia (Academy of Nutrition and Dietetics: AND, 2012;Islam and Indarjit, 2012). Theseare usedin a range of edible products like table top sweeteners, baked products and pharmaceuticals, and have been approved by the United States (US) Food and Drug Administration (FDA) as those that are safe for consumption, hence are now regarded as "Generally Regarded As Safe" (GRAS) with established Accepted Daily Intake (ADI) and Estimated Daily Intake (EDI) for each of them (AND, 2012).

2.6.1.1 Saccharin

Saccharin is the foremost ASs to be discovered and the oldest non-nutritive sweetener to gain approval for use in food and beverage (AND, 2012). Constantine Fahlberg, in 1897 accidentally discovered saccharin from O-methyl-benzenesulfonamide (OCBS),while working in Ira Remsem's laboratory (DuBois, 2006). Rather than an expected O-carboxy-benzenesulfonamine compound, the oxidation of OCBS resulted in a novel heterocyclic compound (1,1-dioxo-1,2-benzothiazol-3-one), also called saccharin. He accidentally spilled a chemical on his hand and later noticed that the chemical has a strong sweet taste,which was later named saccharin.

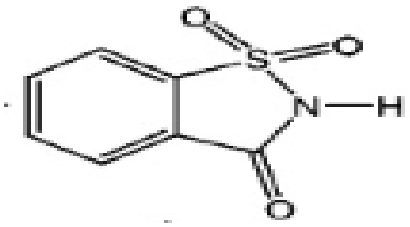


FIGURE 2.5: Molecular structure of saccharin (DuBois, 2006).

Saccharin is water soluble, heat stable and has some brand names like “Sweet N Low” and “Sugar Twin” (AND, 2012). It is about 200 to 700 times sweeter than sucrose and it is not metabolized in human (Mitchell and Pearson, 1991). However it is known to have a bitter after taste that is somewhat metallic. The good stability of saccharin at high temperatureallows it to retain its sweetness when used for cooking and baking. Furthermore, it is rapidly excreted in the feces and urine and also provides no energy, since it is not metabolized by the body (AND, 2012).

Saccharin has been used as a replacement for sugar for several decades and was originally added to the “GRAS” list. However, in 1970’s there were some controversies regarding the safety of saccharin consumption, because it has been reported that saccharin isassociated with the development of cancer of the bladder in laboratory rats. For this reason, the US FDA proposed a ban on the use of saccharin, but was replaced in the same year by an 18 months moratorium, which required product containing saccharin to carry the following warning: “Use of this product may hazardous to health. These products contain saccharin, which has been determined to cause cancer in laboratory animals” (ADIA, 1998). However, several studies on the use of saccharin, especially in higher users like diabetic individuals did not show or support the carcinogenicity of saccharin in human (Morgan and Wong, 1985; Risch*et al*., 1988). The mechanism behind the carcinogenicity of saccharin in rats was later determined by the National Institute for Environmental Health Science (NIEHS) to be irrelevant to human beings but may however increase the risk of adverse effect for sub groups like heavy smokers.

In 1991, the proposed ban of saccharin was formerly removed by the US FDA and in 2000 the warning label requirement for products containing saccharin was formerly repealed (ADIA, 1998; AND, 2012). Today saccharin is widely used in various products like canned fruits,

reduced calorie syrup, sweet toppings, baked foods, pharmaceuticals, beverages and chewing gums, and often used in combination with other sweeteners. The Academy of Nutrition and Dietetics, reported in 2012 that no ADI was presently determined for saccharin. However, an EDI of 0.1 to 2mg per kg body weight has been determined for average and higher users (Neltner*et al*., 2011; Renwick, 2006).

2.6.1.2 Aspartame

Aspartame was discovered accidentally in 1965 by James M. Schlatter (AND, 2012). In an attempt to generate gastrin hormone (a tripeptide) for anti-ulcer assessment, an intermediate product (an aspartyl-phenylalanine methyl ester) was produced, which he accidentally noticed had a sweet taste and later named it aspartame.

Aspartame is a non-saccharide sweetener. It is a methyl ester of aspartic acid and phenylalanine dipepetide that is about 160 to 200 times sweeter than sucrose, and has other brand names such as “Nutra Sweet” and “Equal” (ADIA, 1998). Aspartame is not suitable for cooking or baking, since it is relatively unstable under high temperature and pH(Magnuson *et al*., 2007). It is however encapsulated to increase its heat stability and also extend its use in some commercially baked products (ADIA, 1998).

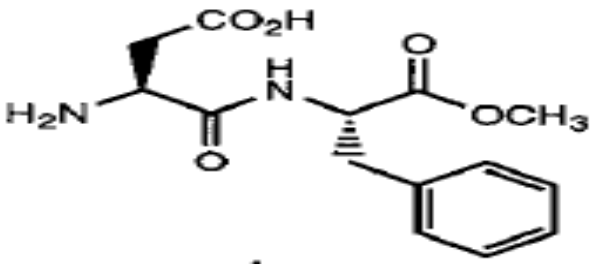


FIGURE 2.6: Molecular structure of aspartame (Ager *et al*., 1998).

Aspartame, like most peptides is hydrolysable to produce constituent amino acids. In the intestine, aspartame is hydrolyzed by intestinal esterases to give aspartic acid methanol and

phenylalanine (Magnuson *et al*., 2007). The amino acids resulting from its hydrolysis can be further metabolized to provide 4kcal/g of energy (Ager *et al*., 1998). However, because of the intense sweetness of aspartame, only a small amount is needed to provide required sweetness, thus only a negligible amount of energy is derived from aspartame.

It has been reported that formaldehyde and formic acid can also be formed from the metabolism of aspartame, which have been found to be the cause of negative side effects such as headache, memory loss, joint pains, muscle sparsm, dizziness, fatigue, vision problem, asthma or chest tightness, rashes and weight gain (Dyar, 2008). Diketopiperazine is another toxic product that can result from the breakdown of aspartame when exposed to heat (ADIA, 1998). However, reports from animal toxicity studies have shown that the small amounts of aspartame used in beverages cannot produce a toxic amount of diketopiperazin (FDA, 1983). Also, in 1995, the US department of health and human services (HHS) reported some cases of persons who complained of allergic reactions like edema of the lips, tongue and throat; skin reactions; and respiratory problems after consumption of aspartame, although when investigated under controlled experimental conditions, aspartame was not able to produce similar allergic reactions in the same individuals (Garriga*et al*., 1991; Geha*et al*., 1993).

Furthermore, because aspartame is hydrolyzed in the intestine to yield aspartic acid and phenylalanine, it is advised that people with the rare hereditary disease, phenylketonuria (PKU) should consume products containing aspartame with caution. Infact, the FDA requires that food containing aspartame should carry the following label: "Phenylketonurics: contains phenylalaninine" (AND, 2012). Phenylketonuria is a rare homozygous recessive in-borne error of metabolism, in which sufferers are unable to metabolize phenylalanine (ADIA, 1998); and as such, when such people consumephenylalanine, it causes increased plasma levels of phenylalanine (Stegink and Filer, 1996), deranged monoamine neurotransmitters synthesis (Maher and Wurtman, 1987) and adverse effects (Wolf-Novak *et al*., 1990).

In 1981 and 1983, aspartame received approval by the FDA to be used in specific foods and soft drinks respectively, and in 1996, it gained approval as a general use sweetener (AND, 2012). It is presently used in more than 6000 products marketed around the world, including diet sodas, sugar-free yogurts, ice-cream, chewing gums, gelatin powder etc. However, despite the use of aspartame in numerous products, the average consumption for highest users still remains below

the recommended ADI (Magnuson *et al*., 2007; Renwick, 2006). The recommended ADI and EDI of aspartame are about 50mg/kg body weight and 0.2 to 4.1mg/kg body weight respectively (Magnuson *et al*., 2007).

2.6.1.3 Sucralose

Sucralose (trichlorogalactosucrose) is a halogenated disaccharide sweetener, in which three hydroxyl groups in the sucrose molecule are replaced by three chlorine atoms (Ager *et al*., 1998; Grotz and Munro, 2009). It was accidentally discovered in 1976, while testing for halogenated sugar in a British sugar company called Tate and Lyle (Molinary and Quinlan, 2006).

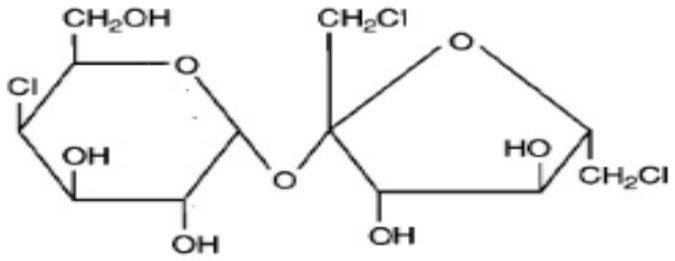


FIGURE 2.7: Molecular structure of sucralose (Molinary and Quinlan, 2006).

Sucralose is about 600 times sweeter than sucrose, heat stable and suitable for cooking and baking (ADIA, 1998). It has brand names like “Splenda” and “Sucra Plus”. Although sucralose is synthesized from sucrose, a rapidly digested and absorbed sugar, it is not rapidly metabolized or absorbed in the body (ADIA, 1998;Molinary and Quinlan, 2006). In fact, AND(2012) reported that about 85% of ingested sucralose is not absorbed by the body, but excreted in the urine and feces unchanged.

The FDA approved sucralose in 1998 as a table top sweetener and also for use in some products like non-alcoholic beverages, desserts and some confections after several review of so many studies in human and experimental animals, which concluded that sucralose is not associated with any risk of cancer and reproductive or neurologic problems (ADIA, 1998). Presently,

sucralose has an FDA approved ADI and EDI of 5mg/kg body weight and 0.1 to 2.0mg/kg body weight respectively, and is used in common products like baked foods, reduced calorie yogurts, canned fruits, flavored creamers etc (AND, 2012).

2.6.1.4 Neotame

Neotame is chemically named N-{N-(3,3-dimethylbutyl)-L-2-aspartyl}-L-phenylalanine 1-methyl ester. Like aspartame, it is a derivative of the dipeptide of phenylalanine and aspartic acid (AND, 2012). After a long term research that was aimed at discovering a high intensity sweetener with good performance and safety qualities, Claude Nefre and Jean Marie developed neotame (Witt, 1999). Althoughneotame is a modified form of aspartame, it however differs from aspartame due to the presence of a neohexyl group and an extended 3,3-dimethyl butyl group that is attached to the amino group of aspartic acid moiety (Indarjit, 2011; Witt, 1999). It is believed that the extended 3,3-dimethylbutyl moiety in neotame inhibits the hydrolysis of the dipeptide unit into aspartic acid and phenylalanine by inhibiting peptidase action (Mayhew *et al*., 2003). Neotame is therefore considered to be safe for consumption even by sufferers of PKU.

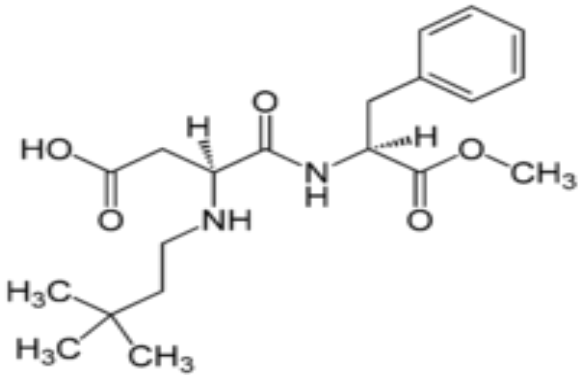


FIGURE 2.8: Molecular structure of neotame (O'Donnell, 2006).

Neotame is about 30 to 60 times and 7,000 to 13,000 times sweeter than aspartame and sucrose respectively and also possesses excellent stability (AND, 2012; Witt, 1999). Witt(1999) further

reported the presence of an N-alkyl function in neotame molecule, which prevents the formation of diketopiperazine products. This quality of neotame and its inertness to some food components like flavoring agents and reducing sugars is responsible for its excellent stability, which is useful in baking and cooking. However, the stability of neotame can vary with pH in aqueous conditions and also with storage conditions (Nofri and Tinti, 2002).

Although neotame is absorbed partially in the intestine, it is however rapidly metabolized by esterases in the body to produce equimolar amounts of de-esterified neotame and methanol (O'Donnell, 2006). The de-esterified neotame is rapidly excreted in the urine and feces, while the amount of methanol produced is very insignificant, thus making it a safe non-caloric sweetener (Norfri and Tinti, 2002). Furthermore, the amount of phenylalanine from neotame that is released into the body is very negligible, since very little amount of neotameis needed to achieve sweetness.

Neotame was approved by the FDA in 2002 as a general use sweetener with an ADI and EDI of 18mg/kg body weight and 0.05 to 0.17mg/kg body weight. Nevertheless, neotame to date is rarely used in foods (AND, 2012). It is however contained in products like sugar free beverages, chewing gums, soft drinks, jams, baked food etc.

2.6.1.5 Acesulfame Potassium (Ace-K)

The discovery and development of acesulfame potassium (Ace-K) was possible, because of the prior discovery of oxathiazonedioxides (OTZDs). It was observed that most methyl and ethyl substitute OTZDs have sweet tastes, and among this group of organic acids, Ace-K was found to have pure sweet taste compared to others (Ager *et al*., 1998).

Ace-K is a combination of an organic acid and potassium that is water soluble and non-toxic. It has other brand names like "Sunnet", "Ace-K" and "Sweet One". Ace-K is about 200 times sweeter than sucrose, and is commonly used in combination with other non-nutritive sweeteners (NNSs) in different products (AND, 2012). It combines well with other NNSs to cause a sweetness synergy that will result in a better sweet taste (AND, 2012; Haber *et al*., 2006). It is relatively heat stable and suitable for cooking and baking.

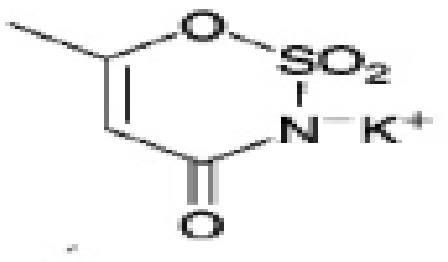


FIGURE 2.9: Molecular structure of acesulfame-K (Harber *et al*., 2006).

Ace-K is not metabolized in the body on ingestion, and about 75% is excreted in the urine and feces chemically unchanged; thus, it provides no energy or calorie to the body (Renwick, 1986).The International Food Information Council Foundation (IFICF) in 1998 reported that over 90 different studies on the safety of Ace-k consumption have indicated that it is not linked to any major life threatening ailment, and it is safe for consumption by any group of individual. According to them, even the amount of potassium derived from Ace-K (about 10mg per ADI) compared to about 2000mg to 3000mg obtained daily from fruits and food consumption is quite negligible to cause any health problem.

Ace-K was approved by FDA in 1998 as a table top sweetener and for use in foods and beverages, and in 2003, as a general use sweetener with an ADI and EDI of 15mg/kg body weight and 0.2 to 1.7mg/kg body weight (AND, 2012). It is used in products like baked foods, chewing gums, syrups, sweet toppings sugar free and frozen desserts etc.

2.6.1.6 Stevia

This sweetener comprises of some extracts from the leaves of the plant *Stevia rebaudianabertoni*, first botanically described in 1899 by a botanist M. S. Bertoni as a sweet perennial herb that was native to South America (Thomas and Glade, 2010). Stevia plant has been for long used to enhance taste of tea and medicinal portion or even chewed because of its sweet taste. However, after stevia plant became popularly known for its sweet leaf, it has been

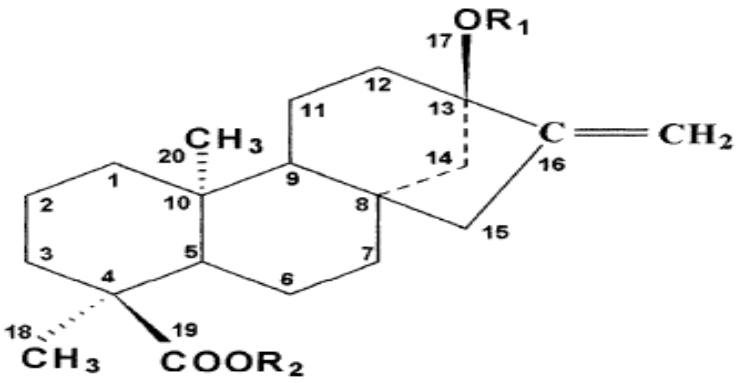
cultivated in China, South East Asia and India, which are some of the major cultivators of Stevia plant in the international market (Mishra, 2011).

FIGURE 2.10: *Stevia rebaudiana*plant (Rajesh *et al*., 2010).

Stevia is believed to bethe major high intensity and non-nutritive sweetener that is non-synthetic and naturally sourced (Megedi*et al*., 2005;Soejarto *et al*., 1983). Stevia leaf contains several extract that are believed to be the cause of its sweet taste. These extracts contain some sweet tasting diterpenoid glycosides, including steviosides, rebaudioside(reb) A, B, C, D and E, dulcoside, steviobioside, Isosteviol and dehydroisosteviol, which have sweetness power ranging from 50 to 450 times compared to sucrose (Chatsudthipong, 2009). These diterpenoid glycosides share a common steviolaglycone backbone, but differ in their carbohydrate residue at carbon-13 and -19, which is believed to be the cause of their relatively different sweetness power (Chatsudthipong, 2009). Steviol glycosides are shelf and heat stable, suitable for cooking and baking, and also possess clean sweet taste, but may be bitter at higher amounts (Goyal*et al*., 2010;Prakash*et al*., 2008).

The adverse health issues associated with other synthetic NNSs greatly influenced the popularity and use of stevia. For instance, the marketing of steviosides in Japan began in the 1970's, when chemically synthesized sweeteners were banned from use (Thomas and Glade, 2010). Since then, stevia plant has been cultivated and used in other places including the US, Canada and Europe

(Chatsudthipong, 2009). Furthermore, even some recent studies have shown that stevia plant contains some phytochemicals that possess hypoglycemic and antibacterial properties (Mishra, 2011).



Compound		R_1	R_2
Stevioside	(1)	-glc-^{2}-glc	-glc
Steviolbioside	(2)	-glc-^{2}-glc	-H
Rebaudioside A	(3)	glc-^{3}-glc-^{2}-glc	-glc
Rebaudioside B	(4)	glc-^{3}-glc-^{2}-glc	-H
Rebaudioside C	(5)	glc-^{3}-glc-^{2}-rha	-glc
Dulcoside A	(6)	-glc-^{2}-rha	-glc
Steviol	(7)	-H	-H

FIGURE 2.11: Chemical structures of the main *S. rebaudiana* sweeteners and their aglyconsteviol (Gardana *et al*., 2003).

Among the steviol glycosides, reb A became the first to be approved as GRAS in 2008, which was then followed by stevioside (AND, 2012). Furthermore, in 2009, the French government approved the use of stevia extract consisting of at least 97% rebA in food and beverages (Thomas and Glade, 2010). Today, the respective ADI and EDI of 4mg to 5mg/kg body weight and 1.3mg to 3.4mg/kg body weight have been set for steviosides, reb A and other steviol

glycosides (AND, 2012; Thomas and Glade, 2010). They are used in several food products like cereals, table top sweeteners, beverages, energy bars etc.

Despite the wide application of artificial or synthetic sweeteners, the health issues associated with their consumption still remains a concern to most people. Some recent studies have reported life threatening side effects that are caused by short- or long-term consumption of most chemically synthesized sweeteners. Saccharin have been reported to have carcinogenic effects on both human and experimental animals (Bell *et al*., 2002; Cohen, 2001), although this is still a controversy (Abergaz, 2007; Huff and LaDou, 2007).

Aspartame has been reported to cause bladder and brain cancer, chronic fatigue, leukemia, lymphomas and some genetic disease (Whitehouse *et al*., 2008). It has also been reported that aspartame can be responsible for fibromyalgia, gastric and pancreatic cancer as well as reduction of anti-oxidant status of the liver (Abhilash*et al*., 2011; Bosetti*et al*., 2009; Ciappuccini*et al*., 2010). As previously stated, aspartame can form toxic substances like formaldehyde, which have been found to cause several negative side effects (Dyar, 2008; Gilli*et al*., 2008; Jacob and Stechschuite, 2008). Furthermore, the fact that phenylalanine is a major product from aspartame breakdown is also a serious problem to individuals suffering from PKU, who cannot metabolize phenylalanine.

Furthermore, sucralose has been reported to adversely affect microflora in the gastroinstestinal tract and cause inflammatory disease of the bowel (Brusick*et al*., 2009; Qin, 2011). In fact, a recent study reported that twelve week daily consumption of 5mg/kg body weight of sucralose reduced the beneficial fecal microflora amounts and adversely alters the expression levels of some drug metabolizing factor (Abou-Donia*et al*., 2008). A number of studies have also reported that sucralose can trigger migraine and headaches (Grotoz, 2008; Hirsch, 2007; Patel *et al*., 2006). However, these adverse effects of sucralose are still a subject of debate (Brusick*et al*., 2009; Gortz and Munro, 2009).

Furthermore, Ace-K has also been reported to be linked to some health problems. Although the total toxicity profile of Ace-k is still yet to be studied (Karstadt, 2010), recent genotoxicity study reported that more DNA damage in albino mice was observed when fed with 150mg to 160mg/kg body weight of Ace-K than when fed with aspartame (Bandyopudhyay*et al*., 2008).

Other than these adverse health issues that have been reported to be associated with the consumption of these chemically synthesized sweeteners, human beings will prefer natural products, in spite of their search for a sugar replacer. Although they have some calorie content and are less sweet compared to chemically synthesized sweeteners, natural sweeteners such as honey, fructose, molasses, high fructose corn syrup, malpe syrup and sugar alcohols have become a preferred sugar replacement in these contexts (Islam and Indarjit, 2012).

2.6.2 Natural and Nutritive Sweeteners

Most natural sweeteners are nutritive and produce calorie, although some of them can also be refined and are not naturally sourced. These refined nutritive sweeteners are also referred to as added sugar and are contained in processed food (ADIA, 1998; AND, 2012). Natural and nutritive sweeteners include cane juice, fruit juice concentrate, honey, glucose, liquid fructose, fruit nectar, crystal dextrose, high fructose corn syrup (HFCS), maple syrup, molasses and sugar alcohols.

Sucrose, glucose and fructose are the primary nutritive sweeteners that occur naturally in food like honey or added as sugars, like in syrups. These sweeteners are present in corn syrups (maple and molasses syrups), pancake syrup, plant nectar, honey and HFCS. HFCS is majorly glucose produced from corn syrup. It is first processed enzymatically to increase its fructose content and then mixed with glucose to contain about 42% to 55% of fructose (AND, 2012). Agave nectar, which is produced from the Agave plant, *Agave tequilana* (Phillips *et al*., 2009) is another natural sweetener that some consumers have shown interest on. Inulin, a fructant present in freshly extracted Agave juice is converted enzymatically from a complex carbohydrate to simple monosaccharide, which contains mostly fructose and some amounts of dextrose and glucose (Cedeno, 1995;Mancilla-Matgalli and Lopez, 2002).

D-tagatose isanother ketohexose nutritive sweetener used in food. D-tagatose is an epimer of D-fructose, which differs from fructose, because of the opposite orientation of the hydroxyl and hydrogen groups at carbon-four (AND, 2012; WHO, 2005). Ithas been identified in gum exudate of cacao tree, but it is obtained from D-galactose by an enzyme-catalyzed isomerization reaction (WHO, 2005). It has similar appearance, texture and sweetness as sucrose but produces lesser

calorie and enhances low postprandial glucose (Levin *et al*., 1995; Livesey and Brown, 1996).D-tagatose is used with other sweeteners in food as a sweetness synergizer, texturizer, stabilizer, humectants, and flavor enhancer.

These nutritive sweeteners improve the functional properties like sensory (e.g. taste), physical (e.g. viscosity), microbial (e.g. preservation) and chemical (e.g. anti-oxidation) characteristics of the food in which they are present (Davis, 1995). They afford approximately similar sweetness, but produce lesser amount of energy or caloriecompared to regular table sugar or sucrose. Furthermore, their poor absorption and rapid removal from the blood (for example fructose) can also improve glycemic control than sucrose or sucrose based sweeteners (Uusitupa, 1994).

However, it has been reported that the chronic or over-consumption of the above-mentionednatural nutritive sweeteners may cause severe health problems such as gastrointestinal discomforts, over-weight, hypertension, obesity, T2D, cardiovascular diseases and other diseases related to metabolic syndrome (ADIA, 1998; AND, 2012; Islam and Indarjit, 2012; Stanhope, 2011). Although not consistently observed (Truswell, 1994), high fructose intake can increase lipid precursorsproduction and risk of hypertriglyceridemia (ADIA, 1998). Furthermore, a parallel rise in obesity, diabetes and hypertension have been witnessed for the past thirty years with fructose consumption (Johnson *et al*., 2007; Segal *et al*., 2007), and several studies have reported that fructose can induce insulin resistance, hyperlipidemia, high blood pressure, oxidative stress, endothelial dysfunction, glomerular hypertension, renal injury and fatty liver disease (Brown *et al*., 2008;Ouyang*et al*., 2005;Sanchez-Lozada*et al*., 2008).

On the other hand, sugar alcohols or polyols are preferred sugar substitutes compared to other natural and artificial sweeteners, because they offer less calorie; other potential health benefits like reduced glycemic response; and less health harzards and risk of dental caries (ADIA, 1998; Islam, 2011).

2.6.3 Polyols or Sugar Alcohols

Sugar alcohols (SAs) can be referred to as sugar replacers, because they are usually used insteadof sugar or sweeteners (McNutt and Sentki, 1996). Most of them occur naturally in plant

products like fruits and berries; although commercially, they are not naturally sourced but synthesized. Commercially synthesized sugar alcohols are mostly produced by hydrogenation of their corresponding carbohydrate precursors to form a distinct compound that is neither a sugar nor an alcohol. They differ from their carbohydrate precursors, because of the presence of a Hydroxyl group (-OH), which replaces the aldehyde (-CHO) or carbonyl (-C=O) functional group of the aldose and ketose moiety of their carbohydrate precursors.

Polyols or SAs have been used for long in several food products in order to reduce carbohydrate intake that can cause blood glucose rise. Some of them are often used in combination with other sweeteners, because of their bulking properties, hence are referred to as bulk sweeteners (AND, 2012; Dills, 1989). Most of them are slowly and partially absorbed in the small intestine by passive diffusion, while the remaining are metabolized indirectly via fermentative degradation by intestinal flora to produce short-chain fatty acids, which are further metabolized to produce energy (ADIA, 1998; Oku and Nakamura, 2002).

Assuming polyols are completely absorbed, direct metabolism will produce about 4 kcal/g of energy like simple sugars. However, since they are only partially absorbed and indirectly metabolized, they provide lesser energy, hence are referred to as low-energy sweeteners. Nevertheless, the energy provided by different polyols varies because of their difference in digestibility, absorption and metabolism (AND, 2012). Furthermore, the incomplete absorption and indirect metabolism of polyols translates into only a small rise in blood glucose, hence they elicit low glycemic effects and insulinenic response. These beneficial properties have made them potential therapeutic substances for managing diabetes, obesity and metabolic syndrome.

However, most polyols can also cause some side effects like bloating, flatulence, abdominal pains and diarrhea when over-consumed, which is believed to be caused by excess unabsorbed polyols reaching the large intestine (ADIA, 1998; Islam, 2011). Nevertheless, SAs are widely used to produce products like chewing gums, baked food, candies, pharmaceuticals, frozen desserts and yoghurts, table top sweeteners etc. Some widely used SAs, presently in use include xylitol, sorbitol, mannitol, maltitol, lactitol and erythritol.

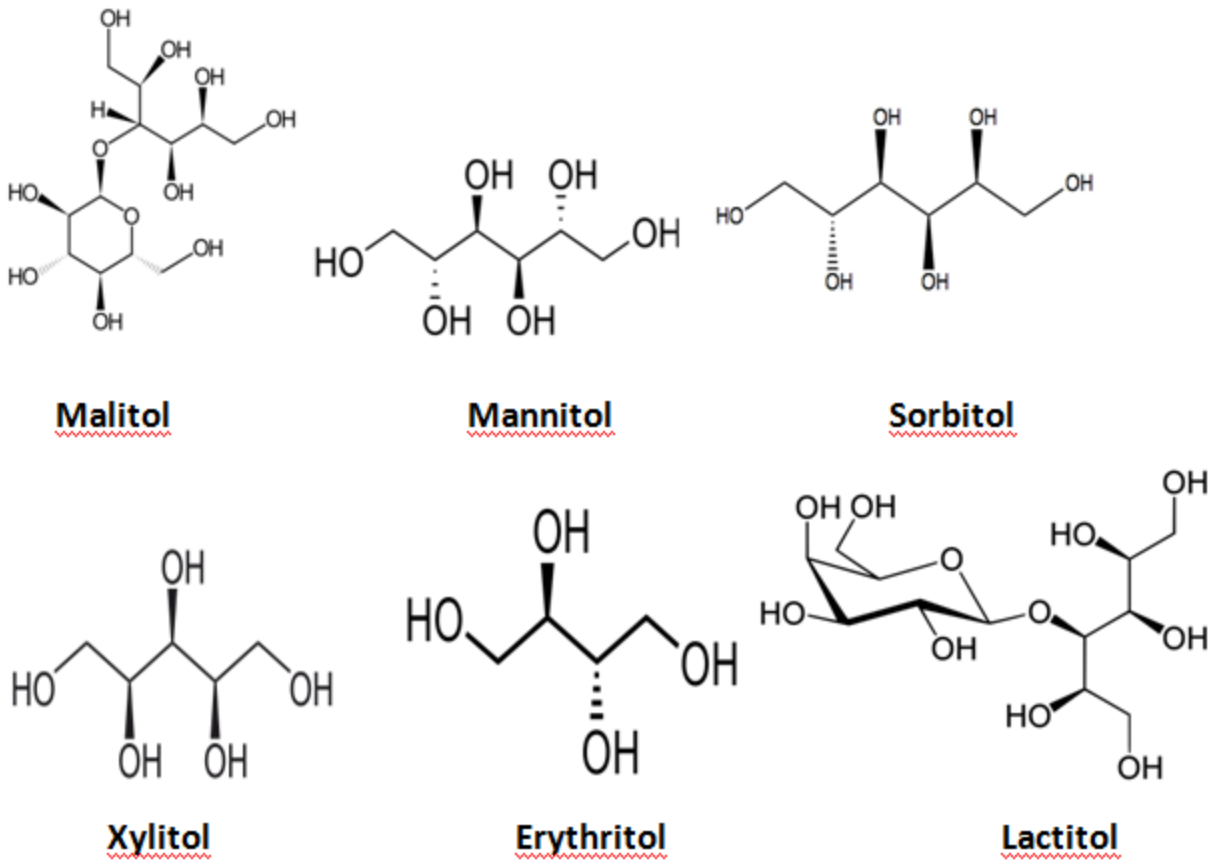


FIGURE 2.12: Molecular structure of the majorly used sugar alcohols (Wikipedia encyclopedia).

2.6.3.1 Mannitol

Mannitol is a sugar alcohol that occurs naturally in pineapples, olives, sweet potatoes and carrots. It appears as a white crystalline substance. Its structure is an optical isomer of sorbitol, with opposite orientations of their hydrogen and hydroxyl group on carbon number two in the carbon back bone of the structure (Figure 2.13). It is typically produced by hydrogenation of fructose (a ketose sugar) derived from starch or sugar (Kearsley and Deis, 2006). It is however commercially extracted in China from a certain species of seaweed. The caloric value of mannitol is 1.6kcal/g and it is about 50% as sweet as sucrose, and also possesses a taste

characterized by a desired cooling effect, often used to mask bitter taste (Kearsley and Deis, 2006;Stowell, 2006).

It has been reported that only about 25% of ingested mannitol is absorbed in the small intestine (Indarjit, 2011). The undigested and unabsorbed portion becomes fermented in the large intestine by colonic bacteria to produce short-chain fatty acid that is metabolized to produce energy. A concomitant release of gases during fermentation may cause abdominal discomforts like pains, boating and flatulence. Mannitol is used for producing products like baked food, pharmaceuticals, candies, chewing gums, chocolates etc.

2.6.3.2 Sorbitol

Being an isomer of mannitol, it is typically produced from the hydrogenation of glucose, an aldose sugar (Kearsely and Deis, 2006). Sorbitol is found naturally in fruits like apples, pears and plums. With a much more cooling effect than mannitol, sorbitol is about 50% to 60% as sweet as sucrose, and is generally considered as a bulk sweetener (Kearsely and Deis, 2006; Stowell, 2006).

Like most sugar alcohols, sorbitol is partially digested and absorbed by the body hence possess the tendency of causing abdominal discomforts. The caloric value of sorbitol is about 2.6kcal/g, and is contained in products like candies, pharmaceutical products, chewing gums, baked food and frozen desserts (Kearsely and Deis, 2006).

2.6.3.3 Erythritol

In the course of a research project initiated by Cerestar to produce different types of polyols, using fermentation process, a strain of yeast was observed to produce erythritol in significant amounts (Perko and DeCock, 2006). Subsequent research showed that erythritol possesses approximately zero caloric value and high digestive tolerance compared to other sugar alcohols (Perko and DeCock, 2006).

Erythritol is a four carbon sugar alcohol with an anhydrous crystalline white appearance, and a granular texture similar to sucrose. Although it is rapidly absorbed in the small intestine, it cannot be metabolized by the body and it is subsequently absorbed in the kidney and then excreted in the urine and feces, thus contributes no caloriewith relatively minimal abdominal discomfort. This quality makes it very useful for diabetic and obese individuals.

Like most sugar alcohols erythritol is a bulk sweetenerwith 60% to 70% sweetness compared to sucrose and it possesses an after taste cooling effect when actively dissolving (Perko and DeCock, 2006). It is contained in products like beverages, chewing gums, chocolates, candies, fondants, lozenges and baked food etc.

2.6.3.4 Lactitol

After the discovery of lactitol in 1920 by J. B. Senderens,a French food chemist, it has been produce by the catalytic hydrogenation of lactose using nickel as a catalyst (Young, 2006). It is a disaccharide sugar alcohol that is composed of sorbitol and galactose. Lactitol is a white crystalline powder with high purity and mild sweet taste that resembles the taste profile of sucrose without any after taste and it is only 40% as sweet as sucrose (Stowell, 2006; Young, 2006).

Once ingested, only about 2% of lactitol is absorbed in the small intestine by passive diffusion, while the undigested portion is fermented in the large intestine and metabolized for energy production. For this reason it has high tendencies of causing abdominal discomforts (Young, 2006). It has been reported that only about 2kcal/g of energy is produced from ingested lactitol, without causing any rise in blood glucose level, thus making it suitable for diabetic and obese people (Stowell, 2006; Young, 2006). Lactitol is contained in products like chocolates, baked food, chewing gum, frozen desserts and pharmaceuticals etc.

2.6.3.5 Maltitol

Like sorbitol and mannitol, maltitol is classified into the sugar alcohol group known as hydrogenated starch hydrolysates (HSH). Maltitol is produced from starch by hydrolysis and catalytic hydrogenation (Kearsley and Deis, 2006). Essentially it is the product obtained from hydrogenation of maltose sugar. Maltitol is a disaccharide that is composed of glucose and sorbitol units.

It shears similar properties with sucrose, and it is about 70% as sweet as sucrose (Kearsley and Deis, 2006;Stowell, 2006). Like most sugar alcohols,maltitol is partially digested, absorbed and metabolized in the body, providing only about 2.4kcal/g of energy (Kearsley and Deis, 2006). Maltitol is used to produce products like chocolates, caramels, baked food, and some dairy products like yoghurts and flavored milk etc.

2.6.3.6 Xylitol

Xylitol is a five carbon polyol that was first discovered in 1891 by Emil Fischer, a German chemist, who later reported this discovery in the same year (Bond and Dunning, 2006). Usually less than 1%, xylitol is known to occur naturally in many fruits and vegetables such as yellow plums, strawberries, raspberries, spinach, lettuce, onion, carrots etc (Bond and Dunning, 2006; Mäkinen and Söderling, 1980).

However, with the progress in research, researchers discovered the feasibility of deriving xylitol from xylose, which became a major success in the production of xylitol. Xylose is mostly produced from xylan (a polymer of xylose), which is contained in hemicelluloses sources like wood chips and corn-cobs (Bond and Dunning, 2006). Xylose is subsequently converted to xylitol by a catalytic hydrogenation process. Further crystallization, centrifugation and drying steps will bring about the crystalline pure form of xylitol (Figure 2.14).

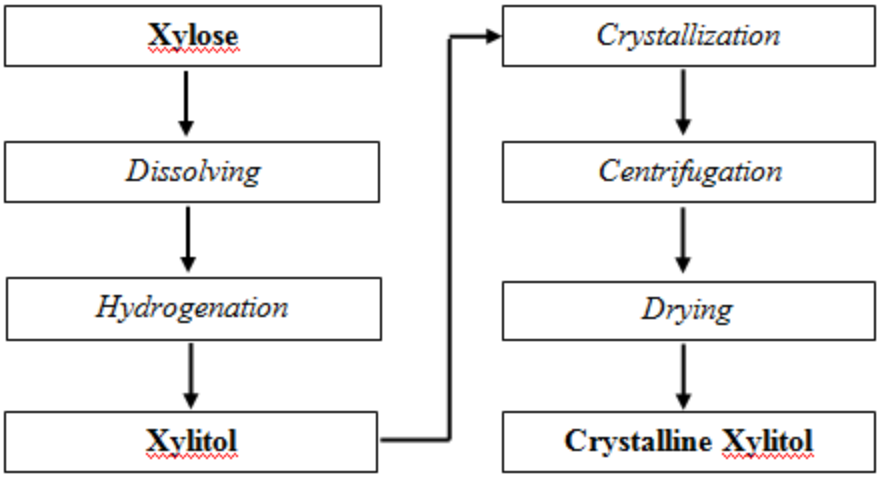


FIGURE 2.13: Outline method for manufacture of xylitol (Bond and Dunning, 2006).

Xylitol appears as a white crystalline solid. It is almost as sweet as sucrose (Stowell, 2006) and gives a taste that is characterized by an after cooling effect void of any bitter after taste; hence it is popularly used in chewing gums (Pierini, 2012). Xylitol is often used in combination with sorbitol and manitol to produce unique sweetness synergy that will enhance its taste (Bond and Dunning, 2006).

Like most sugar alcohols, xylitol is partially and slowly absorbed in the small intestine by passive diffusion. It is reported that only about 25% to 50% of ingested xylitol is digested and absorbed while about 50% to 75% reaches to the large intestine where they are fermented by colonic bacteria to short-chain fatty acids, which is further metabolized for energy production (Bond and Dunning, 2006). Once digested and absorbed, xylitol is withdrawn into the liver, where it becomes part of normal carbohydrate metabolism via the glucoronic acid-pentose phosphate shunt of the pentose phosphate pathway (Figure 2.14) (Bond and Dunning, 2006).

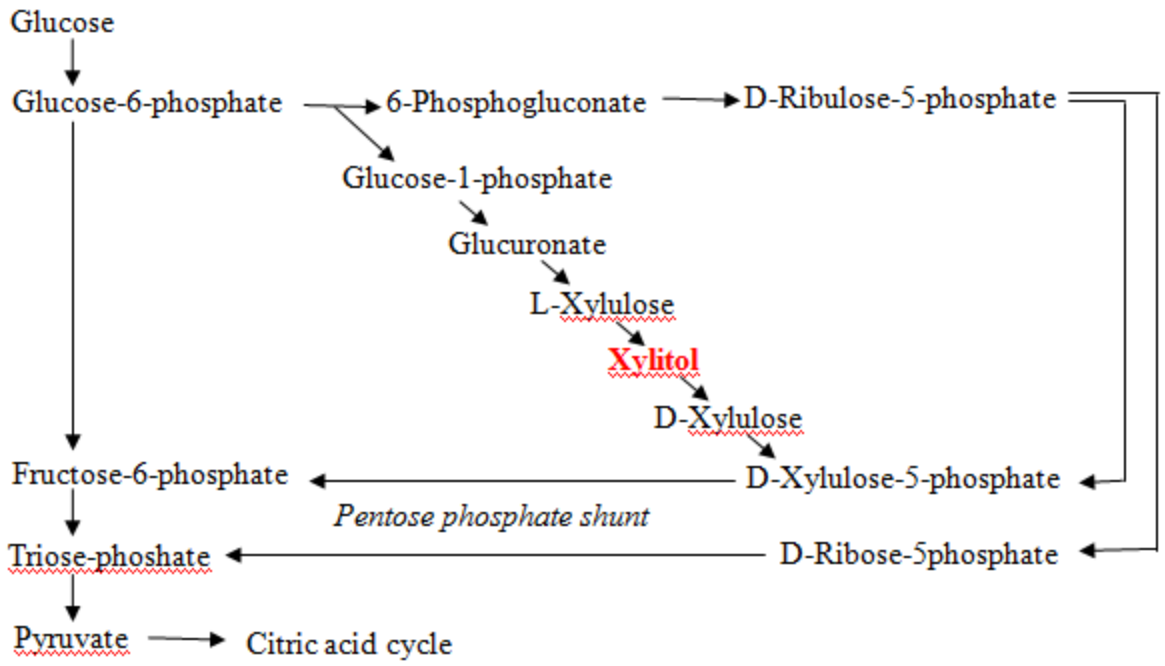


FIGURE 2.14: Glucuronic acid – pentose phosphate shunt. Path-way showing the link between xylitol metabolism (red) and glucuronic acid – pentose phosphate shunt (black) (Bond and Dunning, 2006).

On the other hand, the fermentation of the undigested and unabsorbed portion of the ingested xylitol by colonic bacteria produces gases such as methane hydrogen and carbon dioxide which is responsible for abdominal discomforts like bloating, flatulence and abdominal pains. Furthermore, organic acids resulting from this fermentation process can draw water into the colon via osmosis, which can lead to diarrhea. High quantities of xylitol consumption, over 50g may promote these negative side effects (Bond and Dunning, 2006). However, tolerance ability of xylitol is varied from person to persons.

Despite these few side effects resulting from xylitol consumption, its beneficialeffects cannot be over emphasized. Reports from acidogenicity studies have consistently shown that xylitol cannot be fermented by oral bacteria to produce acid that can cause dental problem like caries development, plaque formation, damaged oral cavities and tooth decay; hence xylitol is referred as being non-acidogenic and non-cariogenic in nature (Bär, 1988). Its ability to completely halt

the progression of caries has promoted its inclusion in oral care products like tooth paste, mouth wash and sugar-free chewing gums (Indarjit, 2011).

Furthermore, another added facet to xylitol's benefit is its suitability for diabetic and obese individuals. The passive and incomplete digestion and absorption of ingested xylitol results in only a small rise in blood glucose that is very insignificant when compared to a similar effect produced by an equivalent amount of sucrose (Bond and Dunning, 2006). This relatively low glycemic index of 13 and the consequent significantly low insulinemic response are important qualities for diabetic consumers. Also the slow and passive absorption of digested xylitol translates into a relatively low caloric value of about 2.5kcal/g (Islam, 2011). This value correlates with the fact that only about 60% of ingested xylitol is effectively metabolized to produce energy, whether through absorption or fermentation (Bond and Dunning, 2006).Supporting this fact, previous studies have reported that xylitol can be a better sweetener than sucrose to manage obesity, diabetes and related metabolic problems and maintain diabetes - related parameters at a physiologically safer and stable condition (Amo*et al*., 2011; Islam, 2011; Kishore *et al*., 2012).

In a recent study, the *in vivo* anti-diabetic effect of xylitol has been reported as well (Islam and Indarjit, 2012). In this study, xylitol significantly reduced food and fluid intake, serum lipids, serum fructosamine, and non-fasting blood glucose; significantly increased serum insulin; and also improved most diabetes-related metabolic parameters in a T2D rat model.In this study, Islam and Indarjit (2012) speculated that xylitol consumption significantly prolonged gastric emptying, decreases food intake and accelerates intestinal transit of nutrients compared to glucose in normal human and experimental animals. This was demonstrated in his study, where lower food and fluid intake were reported in rats fed with 10% xylitol solution compared to the diabetic control. Faster intestinal transit reduces the rate of intestinal nutrient absorption and vice versa. We propose that xylitol may have delayed intestinal absorption of glucose or prevented the digestion of carbohydrate by digestive enzymes such as alpha glucosidase and alpha amylase, thus lowering NFBG and reducing food intake compared to diabetic control rats.

Furthermore, as mentioned above, insulin plays a significant role in the cell uptake of circulating glucose. We also propose that the insulinotropic effect of xylitol, as reported by Islam and Indarjit (2012) may contribute to improving circulating glucose uptake, especially in muscle and

fat cells, hence improved the hyperglycemic condition of the diabetic rats. The above stated mechanisms are possible ways through which xylitol may improve hyperglycemia and anti-diabetic conditions in T2D model of rats. However, studies on the mechanism(s) behind the anti-diabetic effects of xylitol have not been done, especially under diabetic conditions. The present study was therefore aimed at investigating the possible mechanism(s) behind the anti-diabetic effects of xylitol, through the investigation of possible inhibitory effects of xylitol on carbohydrate digestion and glucose absorption under diabetic conditionsas well as the effects of xylitol on *ex vivo*muscle glucose uptake.Considering the wide application and use of xylitol, this study may open up new therapeutic approaches for diabetes and related metabolic disorders that are relatively affordable and void of the numerous side effects associated with most conventional chemically originated drugs.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and reagents

Alpha amylase, alpha glucosidase, streptozotocin, 3,5-dinitrosalicylic acid, di-basic sodium phosphate, paranitrophenyl-α-D-glucopyranoside, acarbose, and citric acid were purchased from Sigma Aldrich, Germany.

Mono-basic sodium phosphate, sodium hydroxide, sodium bicarbonate, sodium chloride, potassium chloride, calcium chloride di-hydrate, mono-basic potassium phosphate, magnesium sulphate, sodium hydrogen carbonate, phenol red, sodium citrate and sodium potassium tartrate were purchased from Merck, South Africa.

Starch, glucose and fructose were purchased from Associated Chemical Enterprise, South Africa, while metformin and NOVO rapid insulin were purchased from a local pharmacy store in South Africa.

3.2 In vitro study

3.2.1 Measurement of In vitro Inhibition of α-amylase Activity

The aim of this assay was to determine possible inhibitory effects of xylitol on α-amylase activity in the hydrolysis of starch. This was determined using methods modified from Mohamed *et al*., 2012. The principle of this assay was based on the fact that reducing sugars resulting from starch hydrolysis by α-amylase enzyme can reduce yellow 3,5-dinitrosalicylic acid (DNSA) to a reddish-brown 3 –amino-5-nitrosalicylic acid (ANSA), which absorbs light at 540nm.

3.2.1.1 Preparation of Reagents

Preparation of 500 mL of assay buffer (20 mM sodium phosphate buffer, pH 6.9): This was prepared as follows:

A 500 mL of 20 mM mono-basic sodium phosphate solution was prepared by dissolving a 1.1998 g of mono-basic sodium phosphate (M.W., 119.98 g/mol) in 500 mL of distilled water (dH_2O)

↓

A 500 mL of 20 mM dibasic sodium phosphate solution was prepared by dissolving a 1.4156 g of dibasic sodium phosphate (M.W., 141.56 g/mol) in 500 mL of dH_2O

↓

A 20 mM mono-basic sodium phosphate solution was steadily added to 20 mM dibasic sodium phosphate solution, while stirring until a pH of 6.9 was attained

↓

This was stored in the refrigerator (4-8 ^{O}C) until further use.

Preparation of 100 mL of 0.4 M sodium hydroxide (NaOH) solution: This was prepared by dissolving 1.6 g of NaOH (M.W., 40 g/mol) in 100 mL of dH_2O.

Preparation of 100 mL of Dinitrosalicylic (DNSA) reagent: This was prepared by using heat to dissolve 1 g of DNSA (M.W., 228.12g/mol) and 30 g of sodium potassium tartrate (M.W., 282.1 g/mol) in 100 mL of 0.4M NaOH.

Preparation of 25 mL of 4 U/mL of α-amylase solution: This was prepared by 7.675 mg of porcine pancreatic α-amylase (1 mg ≡ 13 U) in 25 mL of assay buffer.

Preparation of 50 mL each of 2.5%, 5%, 10%, 20%, 30% and 40% w/v xylitol solutions: This was prepared by dissolving 1.25 g, 2.5 g, 5 g, 10 g, 15 g and 20 g of xylitol in 50 mL of assay buffer respectively.

Preparation of 50 mL of 1% starch solution: This was prepared by dissolving 0.5 g of starch in 50 mL of assay buffer.

3.2.1.2 Assay Procedure

Assay procedure was performed in triplicates as described in the following flow chart:

A 1 mL of sample (2.5% - 40% w/v xylitol solutions) or 1 mL of assay buffer (for control) and 1 mL of 4 U/mL α-amylase solution or 2 mL of assay buffer for blank was added into a test tube, and incubated for 30 min at 37 ^{O}C after mixing well

↓

A 1 mL of 1% starch solution was added and the mixture was incubated for 1 hour at 37 ^{O}C

↓

A 1 mL of DNSA reagent was then added and the mixture was boiled for 10 min

↓

The mixture was allowed to cool and absorbance was read at 540 nm using a UV mini-1240 Spectrophotometer (Shimadzu, Japan).

3.2.1.3 Calculation of Percentage Inhibition of α-Amylase Enzyme Activity

Percentage inhibition was calculated using the following formula:

% Inhibition = [(AbsC – AbsS)/ AbsC] x 100

Where, "AbsC" and "AbsS" mean absorbance of control and samples at 540 nm respectively.

3.2.2 Measurement of In vitro Alpha-Glucosidase Activity Inhibition

The aim of this assay was to determine the possible inhibitory effect of xylitol on the activity of α-glucosidase activity in the hydrolysis of disaccharide or degradation products resulting from starch hydrolysis. This was determined according to methods modified from Wu *et al*., 2012. The principle of this assay was based on the fact that α-glucosidase can hydrolyze paranitrophenyl-α-D-glucopyranoside (PNPG) to release paranitrophenol that absorbes light at 405 nm.

$$\text{PNPG} \xrightarrow{\alpha\text{-glucosidase}} \alpha\text{-D-glucose} + \text{paranitrophenol}$$

3.2.2.1 Preparation of Reagents

Preparation of 500 mL of assay buffer (0.1 M sodium phosphate buffer, pH 6.9): This was prepared as follows:

A 500 mL of 0.1 M mono-basic sodium phosphate solution was prepared by dissolving a 5.999 g of mono-basic sodium phosphate (M.W., 119.98 g/mol) in 500 mL of distilled water (dH_2O)

↓

A 500mL of 0.1 M dibasic sodium phosphate solution was prepared by dissolving a 7.078 g of dibasic sodium phosphate (M.W., 141.56 g/mol) in 500 mL of dH_2O

↓

0.1 M mono-basic sodium phosphate solution was steadily added to 0.1 M dibasic sodium phosphate solution, while stirring until a pH of 6.9 was attained

↓

This was stored in the refrigerator until further use.

Preparation of 500 mL of 5 mM Paranitrophenyl glucopyranoside (PNPG) substrate solution: This was prepared by dissolving 0.07531 g (M.W., 301.25 g/mol) in 50 mL of assay buffer.

Preparation of 25 mL of 1 U/mL α-glucosidase enzyme solution: This was prepared by making up 0.667 mL of 37.5 U/mL *S. cerevisiae* α-glucosidase enzyme solution to 25 mL with assay buffer.

Preparation of 100 mL of 0.2 M sodium bicarbonate (Na_2CO_3): This was prepared by dissolving 2.1198 g of Na_2CO_3 (M.W., 105.99 g/mol) in 100 mL of dH_2O.

Preparation of 50 mL each of 2.5%, 5%, 10%, 20%, 30% and 40% w/v xylitol solutions: This was prepared by dissolving 1.25 g, 2.5 g, 5 g, 10 g, 15 g and 20 g of xylitol in 50 mL of assay buffer respectively.

3.2.2.2 Assay Procedure

Assay procedure was performed in triplicates as described in the following flow chart:

A 0.5 mL of sample (2.5% - 40% w/v xylitol solutions) or 0.5 mL of assay buffer (for control) and 1 mL of 1 U/mL α-glucosidase solution or 1.5 mL of assay buffer for blank was added into a test tube, mixed properly and incubated for 10 min at 25 ^{O}C after mixing well

A 0.5 mL of substrate (5 mM PNPG solution) was added to mixture and incubated for 5 min at 25 ^{O}C

A 2 mL of 0.2 M Na_2CO_3 solution was then added to stop the reaction for 5 min incubation at 25 ^{O}Cand absorbance was read at 405 nm after incubation.

3.2.2.3 Calculation of Percentage Inhibition of α-Glucosidase Enzyme Activity

Percentage inhibition was calculated using the following formula:

% Inhibition = [(AbsC – AbsS)/AbsC] x 100

Where, "AbsC" and "AbsS" mean absorbance of control and samples at 405 nm respectively.

3.3 Ex-vivo Study

3.3.1 Animal Procedures

Five adult male Spagrue-Dawley rats with mean body weight 201.12 ± 12.48 were procured from the Biomedical Resource Center located at the Westville Campus of the University of KwaZulu-Natal, Durban, South Africa. The animals were fasted over-night (12 hours) and euthanized by halothane anesthesia. The abdominal wall was dissected and the whole gastrointestinal tract (GIT) and parts of the psoas muscle were collected and immediately used

for assays. All animal procedures were carried out according to the rules and regulations of the Animal Ethics Research Committee of University ofKwaZulu-Natal, South Africa (Ethical approval number: 097/13/Animal).

3.3.2 Measurement of Glucose Absorption in Isolated Rat Jejunum

The effect of xylitol on glucose absorption by isolated rat intestine was determined using methods modified from Hassan *et al*., 2010.

3.3.2.1 Preparation of Reagents

Preparation of 1 L Kreb's buffer: Kreb's buffer is compose of 118 mM sodium chloride (NaCl), 5 mM potassium chloride (KCl), 1.328 mM calcium chloride dehydrate ($CaCl_2.2H_2O$), 1.2 mM potassium mono-basic phosphate (KH_2PO_4), 1.2mM magnesium sulphate ($MgSO_4$) and 25 mM sodium hydrogen bi-carbonate ($NaHCO_3$). A 1 L of Kreb's buffer was prepared by dissolving 6.896 g of NaCl (M.W., 58.44 g/mol), 0.373 g of KCl (M.W., 74.56 g/mol), 0.188 g of $CaCl_2.2H_2O$ (M.W., 147.02 g/mol), 0.163 g of KH_2PO_4 (M.W., 136.09 g/mol), 0.144 g of $MgSO_4$ (M.W., 120.37 g/mol) and 2.10 g of $NaHCO_3$ (M.W., 84.01 g/mol) in 1 L of autoclaved dH_2O.

Preparation of 500 mL of 11.1 mM glucose Kreb's solution (GKS): This was prepared by dissolving 0.9999 g of D-glucose (M.W., 180.16 g/mol) in 500 mL of Kreb's buffer.

Preparation of 50mL of 3 mM acarbose solution: This was prepared by dissolving 0.09648 g of acarbose (M.W., 645.6 g/mol) in 50 mL of GKS.

Preparation of 100 mL of 10%, 20% and 40% w/v xylitol solutions: This was prepared by dissolving 10 g, 20 g, and 40 g of xylitol in 100 mL of GKS respectively.

3.3.2.2 Assay Procedure

The assay procedure was performed in five replicates as described in the following flow chart:

Five 5 cm length of jejunum was cut from isolated GIT of rats and each jejunum was rinsed by injecting 2 mL of Kreb's buffer through the jejuna lumen using a sterile syringe.

↓

Rinsed jejuna were first inverted to expose their inner wall and villi, and then incubated in carbon (iv) oxide (CO_2) incubation tubes containing 8 mL each of GKS solution (control), 10%, 20% and 40% w/v xylitol solutions and 3 mM acarbose solution (positive control) for 2 hours in a Steri-Cult CO_2 incubator (Labotec, South Africa) at 5% CO_2, 95% Oxygen and 37 ^{O}C condition.

↓

A 1 mL aliquot was collected from each incubation solution before and after incubation and the glucose concentrations were measured in milligram per deciliter (mg/dL) with Thermo Scientific glucose kit in a Labmax Plenno Chemistry Analyzer (Labtest Inc., Costa Brava, Brazil).

3.3.2.3 Calculation of Intestinal Glucose Absorption

This was calculated as the amount of glucose in milligram absorbed per centimeter of rat jejunum using the following formula:

Intestinal glucose absorption = (GC1 - GC2)/ 5cm of jejunum

Where, "GC1" and "GC2" are glucose concentrations (mg/dL) before and after incubation, respectively.

3.3.3 Measurement of Glucose Uptake in Isolated Rat Psoas Muscles

The effect of xylitol on glucose uptake in isolated rat psoas muscles was determined according to methods modified from Abdel-Sattar *et al*., 2012.

3.3.3.1 Preparation of Reagents

Preparation of 50 mL 1 mg/mL metformin solution: This was prepared by dissolving 50mg of metformin in 50 mL of glucose Kreb's solution (GKS).

3.3.3.2 Assay Procedure

The assay was carried out in triplicates as described in the following flow chart:

Three pieces of 0.5 g was cut from the psoas muscles collected from each rat and incubated in CO_2 incubation tubes containing 8 mL each of GKS (control), 10%, 20% and 40% w/v xylitol solutions and 1 mg/mL metformin solution (positive control) for 1 hour in a Steri-Cult CO_2 incubator (Labotec, South Africa) at 5% CO_2, 95% Oxygen and 37 ^{O}C condition. Each 0.5 g of muscle tissue was incubated with and without 100 mU/ mL of insulin.

↓

A 1 mL aliquot was collected from each incubation solution before and after incubation and the glucose concentrations were measured in milligram per deciliter (mg/dL) with Thermo Scientific glucose kit in a Labmax Plenno chemistry analyzer (Labtest Inc., Costa Brava, Brazil).

3.3.2.3 Calculation of Muscle Tissue Glucose Uptake

This was calculated as the amount of glucose in milligram taken up per gram of rat psoas muscle tissue using the following formula:

Muscle glucose uptake = (GC1 - GC2)/ 0.5 g of muscle tissue

Where, “GC1” and “GC2” are glucose concentrations (mg/dL) before and after incubation, respectively.

3.4 In vivo Study

3.4.1 Animals

Thirty-one seven-week-old male Sprague-Dawley rats with mean body weight 222.23 ±13.50 g, procured from the Biomedical Resource Center located at the Westville Campus of the University of KwaZulu-Natal, Durban, South Africa were randomly grouped into five groups, namely normal control (NC), normal xylitol (NXYL), diabetic control (DBC), diabetic xylitol (DXYL) and diabetic acarbose (DBA), which had five animals in each normal animal group and seven animals in each diabetic animal group. The animals were housed in a two-in-one medium-sized poly carbonated cage. The cages were kept in a temperature- and humidity-controlled room with a 12-hour light-dark cycle. All animals were fed with a commercial rat pellet diet and were maintained according to the rules and regulations of the Experimental Animal Ethics Committee of the University of KwaZulu-Natal, South Africa during the entire experimental period (Ethical approval number: 097/13/Animal).

3.4.2 Induction of Diabetes

During the first 2 weeks of the experiment, the animals in the DBC, DXYL and DBA groups were supplied with a 10% fructose solution to induce insulin resistance while the animals in the NC and NXYL groups were supplied with normal drinking water. Thereafter, animals in the DBC, DXYL and DBA groups were intraperitoneally injected with low dose of streptozotocin (40 mg/kg body weight) dissolved in a citrate buffer (pH 4.5) to induce partial pancreatic β -cell dysfunction, while animals in the NC and NXYL groups were injected with a similar volume of citrate buffer only. One week after the streptozotocin injection, the nonfasting blood glucose (NFBG) levels of all animals were measured in the blood collected from the tail vein by using a portable Glucoplus glucometer (Glucoplus Inc., Saint- Laurent, Que., Canada), and animals with a NFBG level $\geq$ 300 mg/dl were considered to be diabetic. Animals with a NFBG level < 300 mg/dl were excluded from the study.

3.4.3 Feeding and Sampling

One week after the STZ injection or induction of diabetes, all animals were fasted overnight (16 hours) with free access to drinking water only. After fasting, the animals in the NXYL, DXYL and DBA groups were administered with a single oral dose of xylitol (1 g per kg body weight for NXYL and DXYL groups) and acarbose (200 mg per kg body weight for DBA group) with glucose (2 g per kg body weight), containing 0.05% (w/v) of phenol red (recovery marker) as stated for each group in the following animal experiment flow diagram, while only glucose with phenol red were administered to NC and DBC group. Animals were then sacrificed by halothane anesthesia exactly 1hour after the dose administration, without any access to drinking water. The whole gastro-intestinal tract was removed as quickly as possible and frozen immediately in liquid nitrogen to prevent the movement of gastro-intestinal contents, and immediately preserved at -30^{O}Cfor subsequent analysis.

3.5.3 Sample Preparation

Samples collected were prepared as follows:

The whole GIT was thawed and divided into eight segments: Stomach (St) 1st, 2nd, 3rd, and 4th quarter of the small intestine (S1, S2, S3, and S4); Cecum (Ce); Proximal and distal half of the Colon (L1 and L2).

↓

The weight of each segment was measured with and without content and the content of each segment was collected by using a syringe to inject 10 mL of cold normal saline (0.9% Sodium Chloride solution) through segments.

↓

The weight of content in each segment was determined by subtracting the weight of segment without content from their respective weights with content.

↓

Both GIT tissue segments and contents were homogenized using an Ultra Turrax Tube Drive Work Station homogenizer (IKA Laboratory equipment, Staufen, Germany) in 10 mL of cold normal saline and centrifuged twice using a Hettich Mikro 200 microcentrifuge (Hettich Lab Technology, Tuttlingen, Germany) at 15,000 rpm for 30 min.

↓

Supernatant was collected in another 2mL micro tube and stored at -20^{O}C for further analysis.

The following analysis or assays were performed on both tissue and content homogenates of GIT segments:

- Determination of glucose concentrations
- Determination of phenol red concentration.

3.6.3 Determination of Glucose Concentration

Glucose concentrations in GIT tissue and content of segments were measured in milligram per deciliter (mg/dL) with a Thermo Scientific glucose kit in a Labmax Plenno Chemistry Analyzer (Labtest Inc., Costa Brava, Brazil), using the principle of the glucose oxidase method. The hydrogen peroxide produced from the oxidation of glucose by glucose oxidase enzyme is reacted with 4-hdroxybenzoic acid and 4-aminoantipyrine in the presence of peroxidase enzyme to form a red quinoneimine dye, in which its absorbance between 460 nm and 560 nm is proportional to the glucose concentration of sample homogenates (Trinder, 1969).

Reaction 1: $\text{Glucose} + O_2 + H_2O_2 \xrightarrow{\textbf{Glucose Oxidase}} \text{Gluconic Acid} + H_2O_2$

Reaction 2: $H_2O_2 + \text{4-HBA} + \text{4-AAP} \xrightarrow{\textbf{Peroxidase}} \text{Quinoneimine dye (red)} + H_2O_2$

Where,

"4-HBA" = 4-Hydroxybenzoic Acid and "4-AAP" = 4-Aminoantipyrine.

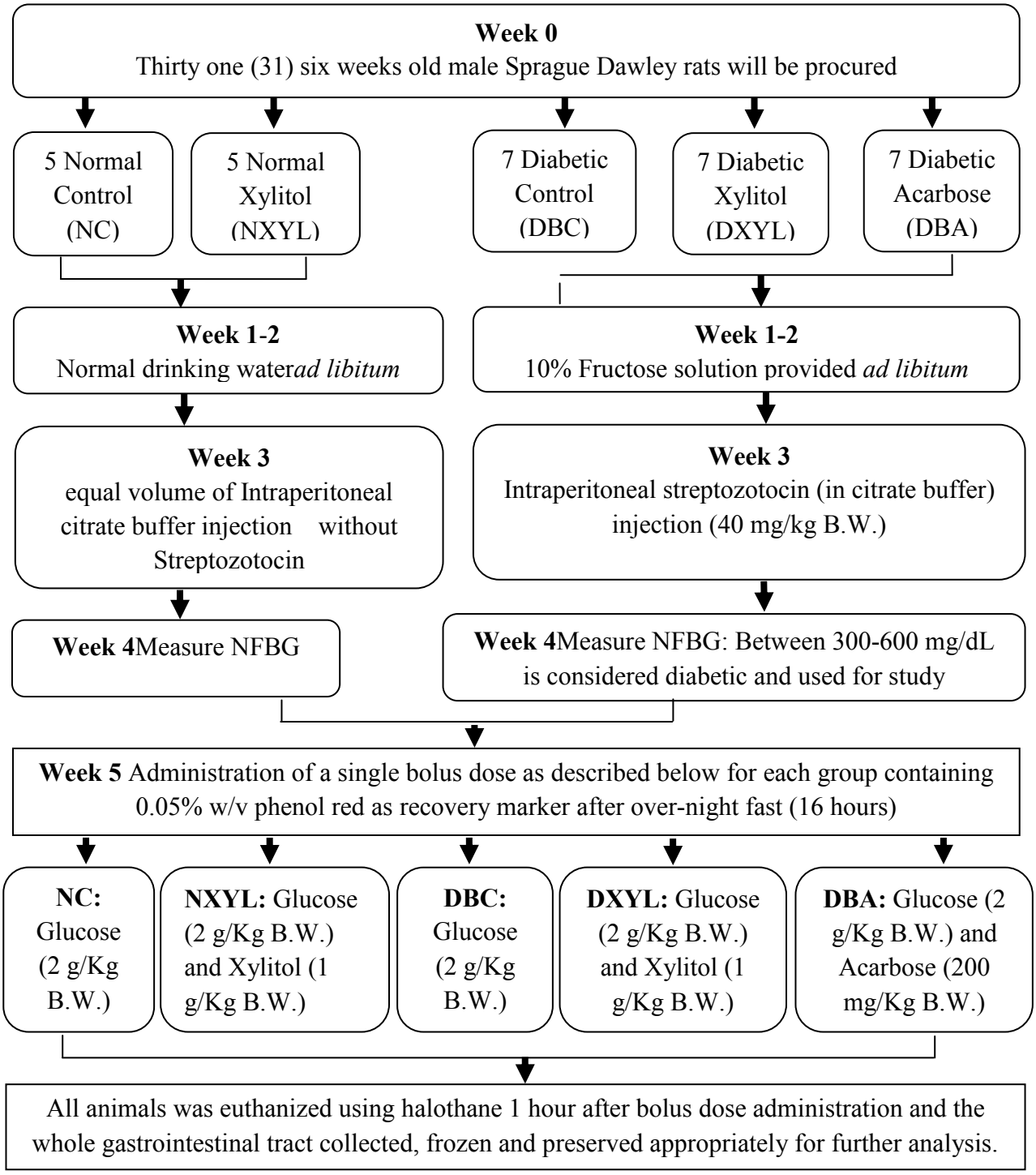


FIGURE 3.1: Flow diagram outlining the experimental design of animal experiment for *in vivo* study.

Amounts of glucose in grams that was given to animals or recovered from segments were calculated using the following formula:

Amount of glucose given (g) = Concentration bolus dose (g/mL) x Volume of bolus dose (mL).

Amount of glucose recovered (g) = [(cT or cC)/ 100,000] x Homogenization volume (mL)

Where,

"cT" = Concentration of glucose (mg/dL) recovered from GIT tissue of segments

"cC" = Concentration of glucose (mg/dL) recovered from GIT content of segments

100000 mg/dL = 1 g/mL.

3.6.4 Determination of Phenol Red Concentration

Phenol red concentration was determined according to methods described by French *et al.,* 1968 with slight modification. The principle of the assay is based on the fact that phenol red in a solution will change from an orange red colour to pink or violet colour under alkaline condition, in which the absorbance at 560 nm is proportional to the concentration of phenol red in the sample solution.

3.6.4.1 Preparation of Reagents

Preparation of 25 mL 0.1 M Sodium Hydroxide Solution: This was prepared by dissolving 0.1 g of sodium hydroxide (M.W., 40 g/mol) in 25 mL of distilled water.

Preparation of 1 liter of 0.1 M Sodium Phosphate buffer (pH, 10.5): This was prepared according to the following chart:

A 1 L of 0.1 M dibasic sodium phosphate solution was prepared by dissolving 14.16 g of dibasic sodium phosphate (M.W., 141.56g/mol) in 1 L of distilled water.

A 0.1 M sodium hydroxide was steadily added to 0.1 M dibasic sodium phosphate solution, while stirring until a pH of 10.5 was attained.

↓

This was stored in the refrigerator until further use.

Preparation of Phenol Red Standard Solutions: The phenol red standard solutions with increasing concentrations of 0.0038%, 0.0018%, 0.001%, 0.0005% and 0.00025% w/v were prepared according to the following chart:

A 5 mL of 0.05% w/v phenol red solution was first prepared by dissolving 0.0025 g of phenol red in distilled water.

↓

This solution was then diluted 10 times to a final concentration of 0.005% w/v by making up 0.5mL of the solution (0.05% w/v) to 5mL with distilled water.

↓

Then, 500µL of phenol red standard concentrations was prepared by making up, 380 µL, 180 µL, 100 µL, 50 µL and 25 µL of 0.005% w/v solution to 500 µL with distilled water to give phenol red standard concentrations of 0.0038%, 0.0018%, 0.001%, 0.0005% and 0.00025% w/v respectively.

3.6.4.2 Assay Procedure

Assay procedure is described in the following flow-chart:

A 30 µL of sample homogenates or phenol red standards or 0.1 M sodium hydroxide solution (for Blank) was added into a 96-well plate.

↓

Then a 210 µL of 0.1 M dibasic sodium phosphate solution (pH, 10.5) was added and mixed gently.

↓

Absorbance was read at 560 nm for phenol red concentration and at 420 nm to correct for bile pigment using a Spectrostar Nano spectrophotometer (Bmg Labtech, Offenburg, Germany).

3.6.4.3 Calculation of Phenol Red Concentrations of Samples

Phenol Red concentrations of samples were calculated as according to the following flow chart:

The absorbance at 560 nm of the phenol red standards was first plotted against their corresponding concentrations using Microsoft Excel, 2007 program to obtain a standard curve.

↓

From the phenol red standard curve, the phenol concentrations of each sample was extrapolated from their respective absorbance at 560 nm.

Amounts of phenol red (PR) in grams that was given to animals or recovered from segments were calculated using the following formula:

Amount of PR given (g) = [Concentration bolus dose (% w/v)/ 100] xVolume of bolus dose (mL).

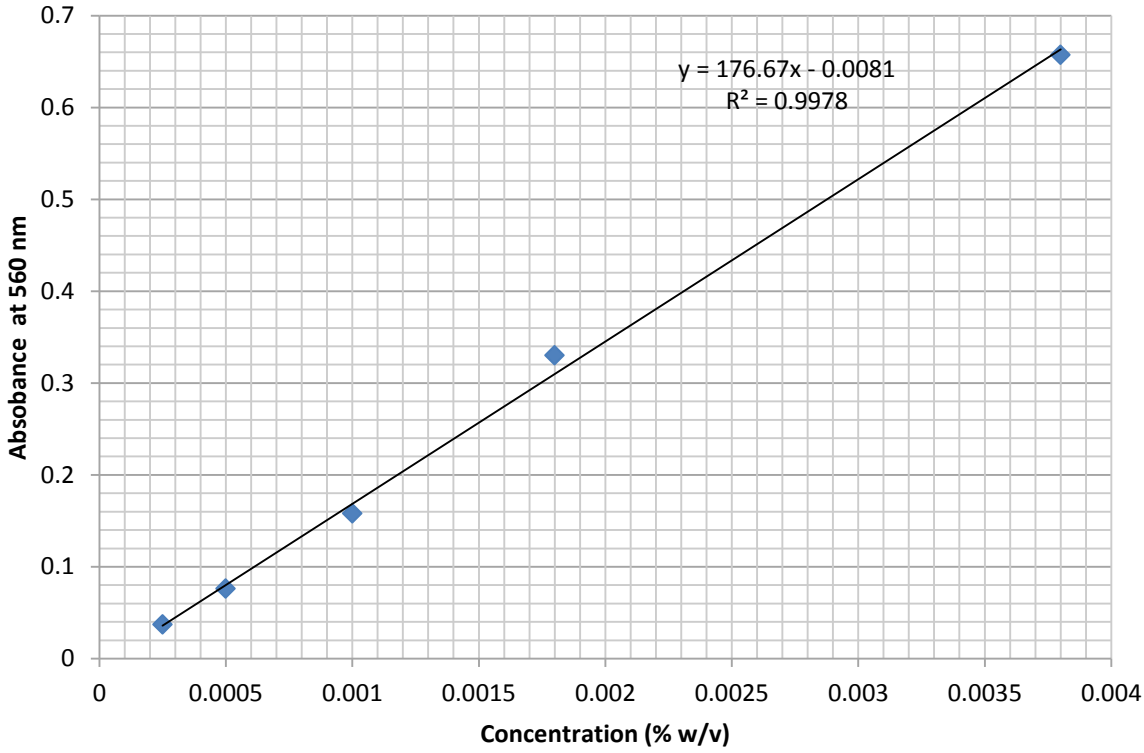
Amount of PR recovered (g) =[(cT or cC)/100] x Homogenization volume (mL)

Where,

"cT" = Concentration of PR (% w/v) recovered from GIT tissue of segments.

"cC" = Concentration of PR (% w/v) recovered from GIT content of segments.

100% w/v = 1 g/mL.



Scale: Y-axis: 0.1 units rep 1cm

X-axis: 0.0005 units rep 1cm

FIG 3.2: Phenol red standard curve: a graph of absorbance of phenol red standard solutions versus standard concentrations.

Percentage recovery of phenol red in GIT tissue or contents of segments was calculated using the following formular:

Percentage recovery in given segment (%) = [(aT or aC)/ Amount of PR given (g)] x 100

Where,

"aT" = Amount of PR (g) recovered from GIT tissue of that particular segment.

"aC" = Amount of PR (g) recovered from GIT content of that particular segment.

Total percentage recovery of phenol red for each animal was equivalent to the percentage phenol red recovered from the entire GIT, which was given by the sum total of the percentage recovery of both tissue and content of all segments.

3.6.5 Calculation of In Vivo Parameters

Amounts of phenol red and glucose recovered from GIT segments were used as indices to calculate the following parameter according to methods described by Islam and Sakaguchi (2006), which were expressed in percentages:

- Gastric emptying of the stomach
- Glucose absorption index (GAI) in the GIT
- Digesta transit along the GIT

3.6.5.1 Calculation of Stomach Gastric Emptying

This parameter denotes the extent or degree to which the stomach empties its content that was ingested. It was calculated using the following formula:

Gastric emptying (%) = $[(A - B)/ A] \times 100$

Where,

"A" = Total amount of PR (g) recovered from the whole GIT

"B" = Total amount of PR (g) recovered from the stomach

3.6.5.2 Calculation of Glucose Absorption Index (GAI)

This denotes the extent to which glucose is absorbed at each segment of the GIT. It is the percentage of glucose passing through a given segment that was absorbed. It was calculated using the following formula:

GAI (%) in a given segment of the GIT $= \left(1 - [(a/b)/(c/d)]\right) \times 100$

Where,

"a" = Amount of glucose (g) recovered from that segment

"b" = Amount of PR (g) recovered from the same segment

" c" = Amount of glucose (g) given to corresponding animal

"d" = Amount of PR (g) given to corresponding animal

3.6.5.3 Calculation of Digesta Transit

This denotes the degree or extent to which GIT content passes from one segment to the next. It is the percentage rate at which GIT content transits along the intestine. The digesta transit at a given segment of the GIT is expressed a percentage ratio of the amount of GIT content leaving that particular segment to the amount reaching the same segment, and it was calculated using the following formula:

Digesta transit in a given segment (%) = (a / b) x 100

Where,

"a" = Amount of phenol red (g) recovered from that particular segment of the GIT to the distal colon excluding the amount of phenol red (g) recovered from that particular segment.

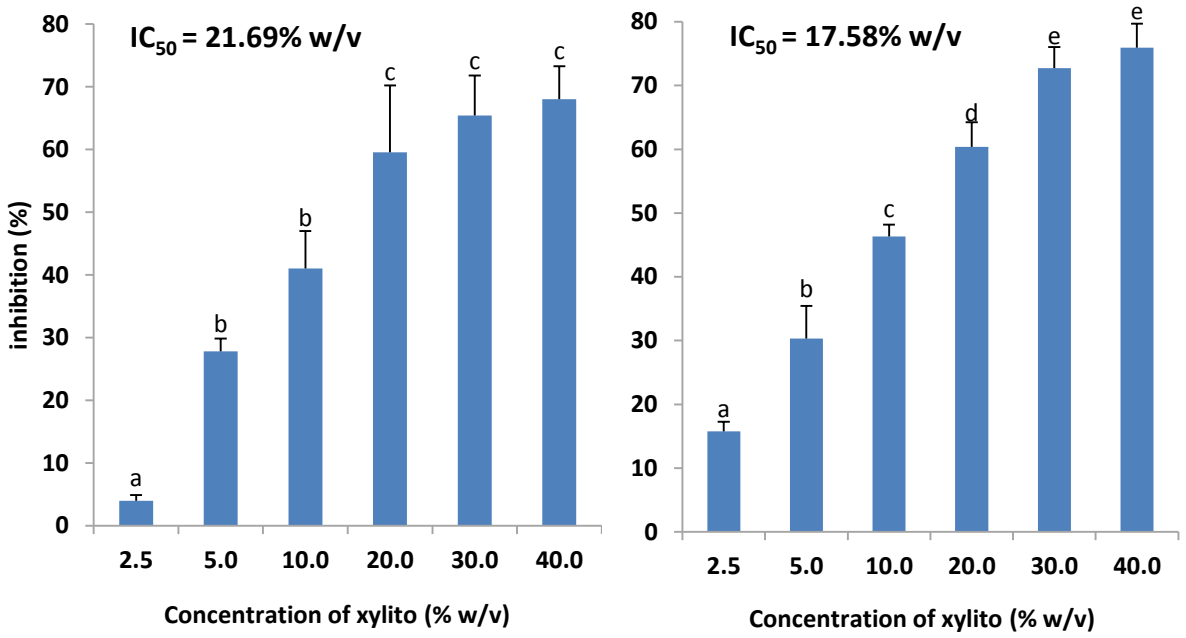
"b" = Amount of phenol red (g) recovered from that particular segment of the GIT to the distal colon.

CHAPTER 4

RESULTS

4.1 Effects of Xylitol on the Activities of Alpha Amylase and Alpha Glucosidase

The data showing the effects of increasing concentrations of xylitol on *in vitro* alpha amylase enzyme activity are represented in figure 4.1a. Result showed that there was an *in vitro* inhibition of alpha amylase enzyme activity in the presence of xylitol compared to the absence of xylitol. However, this inhibition was concentration-dependent. Increasing concentrations of xylitol from 2.5% to 40% caused corresponding increase of enzyme activity inhibition, with IC_{50} value of 21.69% w/v of xylitol (Figure 4.1a). Xylitol concentrations at 40% showed the highest enzyme inhibition, which was relatively higher than concentrations at 20% and 30%, but significantly higher ($p< 0.05$) than concentrations at 2.5%, 5% and 10%, when there was no significant difference between concentrations at 5% and 10% (Figure 4.1a). Lower inhibition was observed at 2.5% concentration of xylitol ($p< 0.05$) compared to all other concentrations



(a) (b)

Figure 4.1: Graph showing the effect of xylitol on activities of alpha amylase **(a)**and alpha glucosidase **(b)***in vitro*. *Data are presented as mean ± SD of triplicates of analysis. Different letters presented above the bars for a given enzyme are significantly different from each other (p < 0.05. Tukey's HSD post-hoc test, IBM, SPSS, version 21).*

Figure 4.1b represents data showing the *in vitro* effects of increasing concentrations of xylitol on alpha glucosidase enzyme activity. Compared to the control, which was without xylitol, all concentration of xylitol showed enzyme activity inhibition. This was also in a concentration-dependent manner, because increasing concentrations of xylitol caused a concomitant increase in enzyme activity inhibition with IC_{50} value of 17.58% w/v xylitol (Figure 4.1b). Enzyme activity inhibition at all concentrations where significantly different from each other ($p< 0.05$), except for between concentrations at 30% and 40%, which both showed the significantly higher ($p< 0.05$) enzyme activity inhibition compared to all other concentrations (Figure 4.1b).

4.2 Effects of Xlitol on Glucose Absorption in Isolated Rat Jejunum

The data showing the effects of xylitol on *in vitro* glucose absorption in isolated rat jejunum are presented in Figure 4.2. The result showed that the amount of glucose absorbed by isolated rat jejunum in the presence of xylitol was concentration-dependent. Amount of glucose absorbed was lowest at 40% xylitol (1.75 ± 0.46 mg/cm jejunum), which was significantly different ($p< 0.05$) from the control (3.27 ± 0.46 and mg/cm jejunum) and concentrations at 10% xylitol (3.27 ± 0.67 mg/cm jejunum), but not significantly different from the amount of glucose absorbed at 20% xylitol (2.90 ± 0.78 mg/cm jejunum) and in 3 mM acarbose (2.40 ± 0.65 mg/cm jejunum) (Figure 4.2).

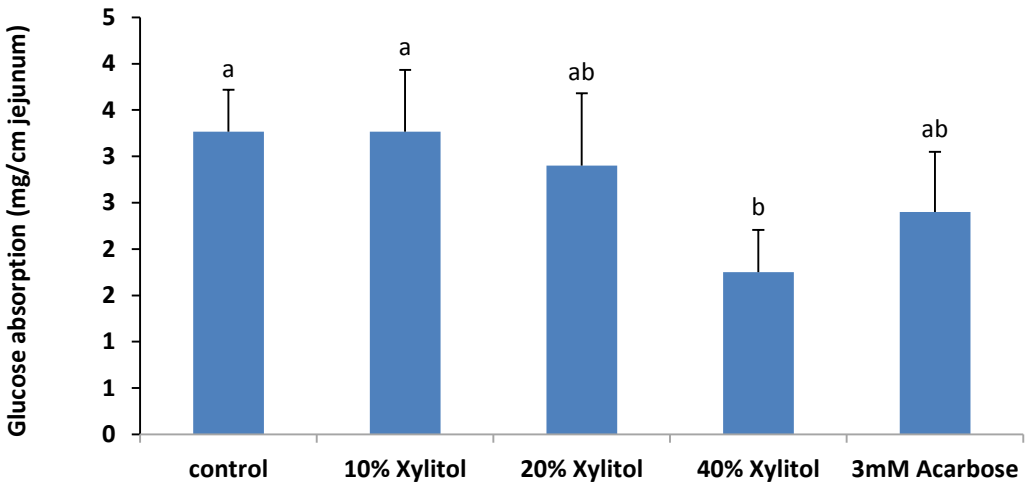


Figure 4.2: Graph showing the effects of xlitol onglucose absorption in isolated rat jejunum*in vitro*. *Data are presented as mean ± SD of five replicates of analysis. Different letters presented above the bars for a given xylitol concentration are significantly different from each other ($p < 0.05$. Tukey's HSD post-hoc test, IBM, SPSS, version 21).*

4.3 Effects of Xlitol on Glucose Uptake by Isolated Rat Psoas Muscle

The effect of xylitol on *in vitro* glucose uptake in isolated rat psoas muscle is presented in figure 4.3. Data showed that the amount of glucose uptake by psoas muscle in the presence of xylitol was also concentration dependent. This was highest at 40% xylitol with and without insulin (54.00 ± 5.29 and 54.70 ± 16.29 mg/g tissue weight respectively), which was significantly different ($p< 0.05$) from the control but not significantly different from the amount of glucose uptake at 10% and 20% xylitol, and 1 mg/mL metformin (Figure 4.3). Although not significantly different, the amount of glucose uptake by isolated muscle tissues was higher at 10% and 20% xylitol concentrations compared to the control, but lower compared to 1 mg/mL metformin. Furthermore, there was no significant difference in the amount of glucose uptake between the incubation with insulin and without insulin for all groups (Figure 4.3).

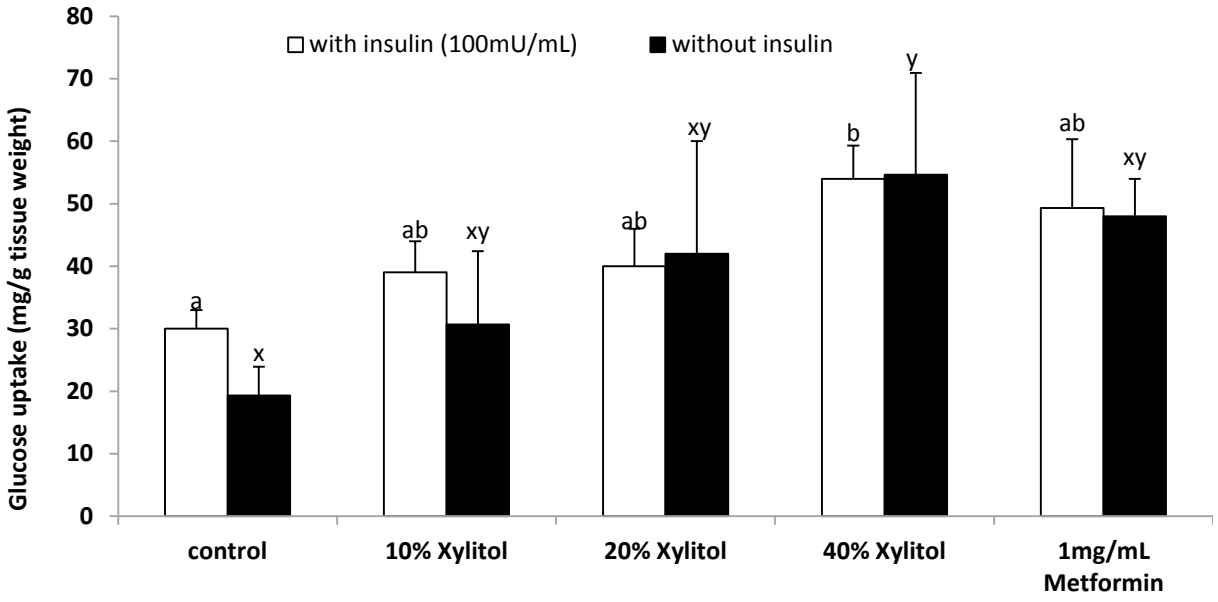


Figure 4.3: Graph showing the effects of xlitol onglucose uptake in isolated rat psoas muscle *in vitro*. *Data are presented as mean ± SD of triplicates of analysis. Different letters ("x" and "y"or "a" and "b") presented above the bars for a given xylito concentration (with or without insulin respectively) are significantly different from each other ($p < 0.05$. Tukey's HSD post-hoc test, IBM, SPSS, version 21).*

4.4 Effects of Xylitol on *In Vivo* Intestinal Glucose Absorption and Related Parameters

The data showing the effects of xylitol on *in vivo* intestinal glucose absorption and other related parameters like nutrient gastric emptying, digesta transit and fluid volume, which can directly or indirectly affect intestinal absorption of glucose, are presented in Figure 4.4 to 4.9. Phenol red was used as a recovery marker to estimate the above mentioned parameters in both normal and diabetic rats. The mean of the total amount of phenol red recovered from the entire gastrointestinal tract(GIT) of all experimental animals was 84.96 ± 10.34%, which was expressed as a percentage of the total amount of phenol red given to the animals.

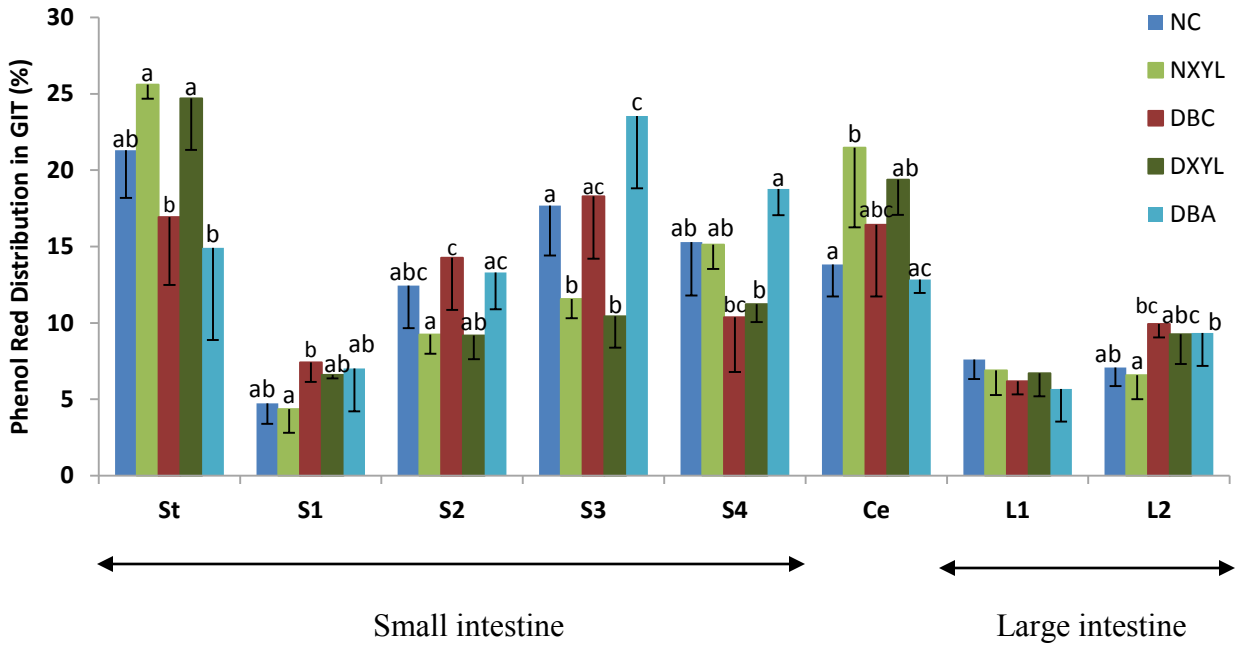


Figure 4.4: Data showing the distribution of phenol red (marker) in the different segments of the GIT at 1 hour after the dose ingestion. *Data are presented as mean ± SD of five to six animals. Different letters presented above the bars for a given segment are significantly different from each other group of animals ($p < 0.05$. Tukey's HSD post-hoc test, IBM, SPSS, version 21).*

The general distribution of phenol red across the different GIT segments was relatively higher in the stomach, distal half of the small intestine and cecum, and lower in the proximal end of the small intestine and colon (Figure 4.4). In the stomach, the phenol red recovered was significantly higher ($p< 0.05$) in the NXYL and DXYL groups compared to the DBC and DBA groups, but only relatively higher compared to the NC group, when there was no significant difference between the NC, DBC and DBA groups. The phenol red distribution of the DBC group was significantly higher ($p< 0.05$) compared to the NXYL group in the 1st quarter of the small intestine, but compared to the NXYL and DXYL in the 2nd and 3rd quarters, when no significant difference was observed when comparing between other groups of the 1st quarter. In the 4th quarter of the small intestine and distal half of the colon, there was no significant difference in phenol red distribution between the NC and NXYL groups as well as between the DBC and DXYL groups, where as in the cecum, the phenol red distribution of the NXYL and DXYL groups was relatively higher compared to the NC, DBC and DBA groups, when no significant

difference was observed when comparing between all groups in the proximal half of the colon (Figure 4.4).

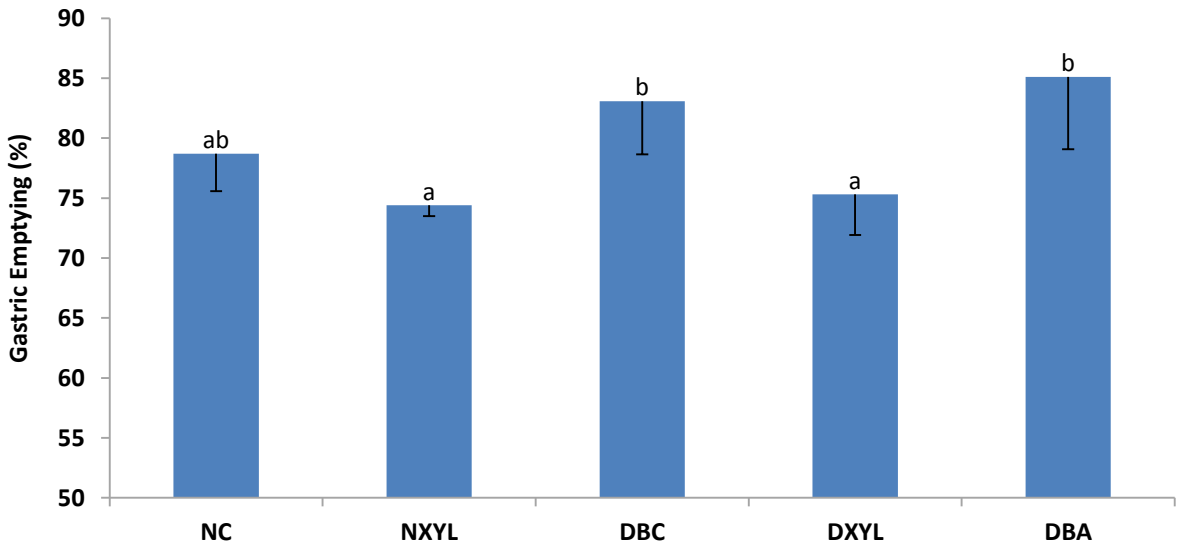


Figure 4.5: Data showing the gastric emptyingat 1 hour after the dose ingestion. *Data are presented as mean ± SD of five to six animals.Different letters presented above the bars for a given group are significantly different from each other group of animals ($p < 0.05$. Tukey's HSD post-hoc test, IBM, SPSS, version 21).*

The data showing the gastric emptying of the stomach for all groups are presented in Figure 4.5. The gastric emptying of the NXYL and DXYL groups was significantly lower (p<0.05) compared to the DBC and DBA groups, when no significant difference was observed between the NC and all other groups. However, the gastric emptying of the NC group was relatively higher compared to the NXYL and DXYL groups, but relatively lower compared to the DBC and DBA groups (Figure 4.5).

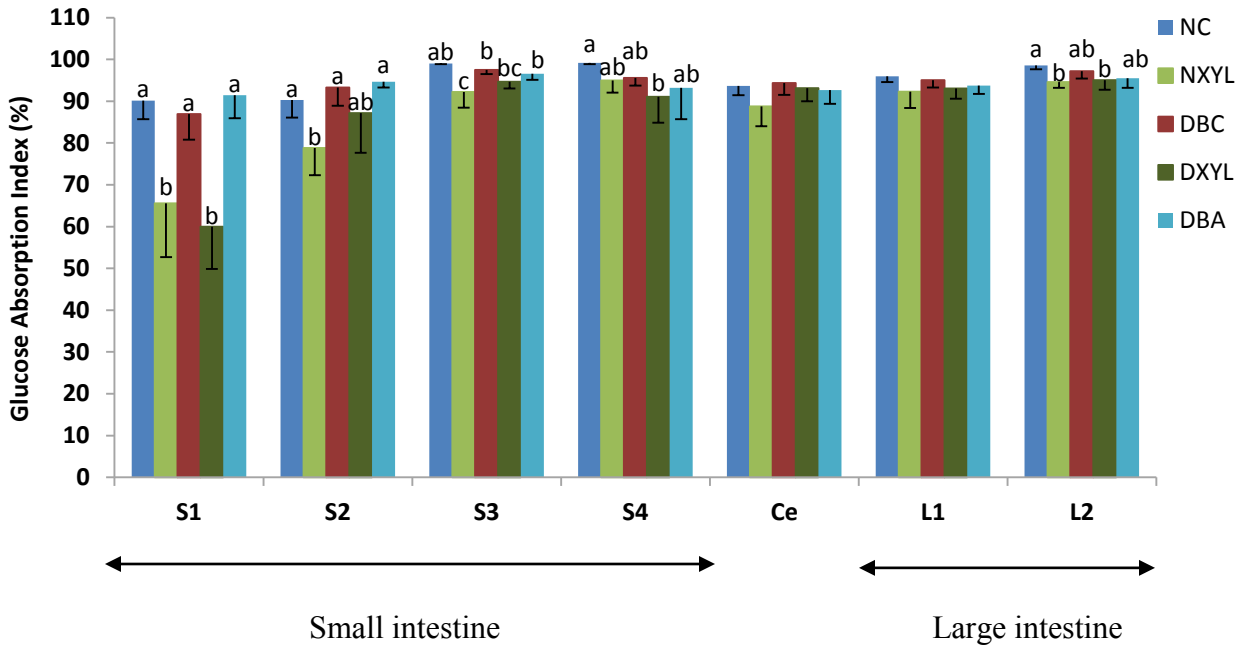


Figure 4.6: Data showing the glucose absorption index (GAI) in the different segments of the GIT at 1 hour after the dose ingestion.*Data are presented as mean ± SD of five to six animals. Different letters presented above the bars for a given segment are significantly different from each other group of animals ($p < 0.05$. Tukey's HSD post-hoc test, IBM, SPSS, version 21).*

The data showing the intestinal glucose absorption index (GAI) are presented in Figure 4.6. In the 1st quarter of the small intestine, the GAI of the NXYL and DXYL groups was significantly lower ($p< 0.05$) compared to the NC, DBC and DBA groups, when there was no significant difference between NC, DBC and DBA groups, as well as between the NXYL and DXYL groups. In the normal rats, the GAI of the NXYL group was significantly lower ($p< 0.05$) in the 2nd and 3rd quarters of the small intestine, and distal half of the colon, but insignificantly lower in the last quarter of the small intestine compared to the NC group. In the diabetic animals, the GAI of the DXYL group in all these segments was relatively lower compared to the DBC and DBA groups. In the 1st quarter of the small intestine, the GAI was significantly lower ($p< 0.05$) in the DXYL group compared to the NC, DBC and DBA groups. However, in the 3rd and last quarters of the small intestine, and distal half of the colon, the GAI of the DXYL group was significantly lower ($p< 0.05$) compared to the NC group, when no significant difference was observed between similar groups in the 2nd quarter of the small intestine (Figure 4.6). No significant

difference was observed in the cecum and proximal half of the colon when comparing between all groups.

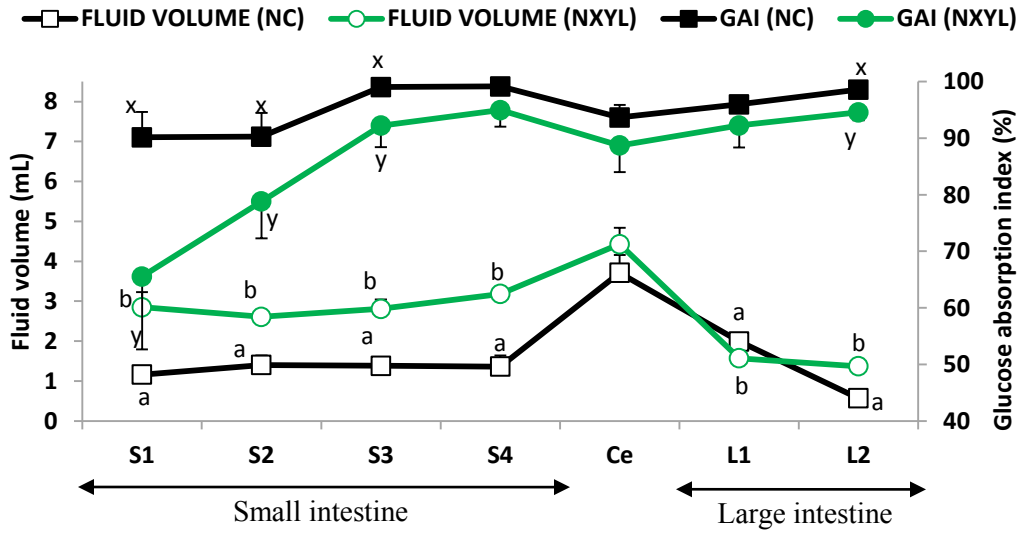
Table 4.1: Data showing the percentage of digesta transit in the different segments of the GIT during 1 hour of experimental period.

GROUP	S1	S2	S3	S4	Ce	L1
	← Small intestine →					← Large intestine →
	Digesta Transit (%)					
NC	93.38 ± 1.77	83.23 ± 3.32	71.25 ± 5.28^{a}	65.56 ±5.82	51.71 ± 3.73^{a}	48.53 ± 8.09^{a}
NXYL	94.53 ± 2.54	86.94 ± 2.20	81.06 ± 2.03^{b}	69.20 ± 4.72	38.98 ± 8.13^{b}	49.40 ± 6.20^{a}
DBC	91.11 ± 1.63	82.65 ± 5.49	70.98 ± 4.83^{a}	71.68±14.15	48.61 ±6.17^{ab}	58.61 ± 8.10^{ab}
DXYL	91.54 ± 0.17	84.24 ± 4.14	81.16 ± 4.42^{b}	75.02 ± 4.12	41.31 ± 3.75^{b}	54.40 ± 4.55^{ab}
DBA	91.77 ± 3.20	84.33 ± 5.68	64.45 ± 6.29^{a}	69.55 ± 9.89	55.5 ± 5.53^{a}	64.8 ± 13.91^{b}

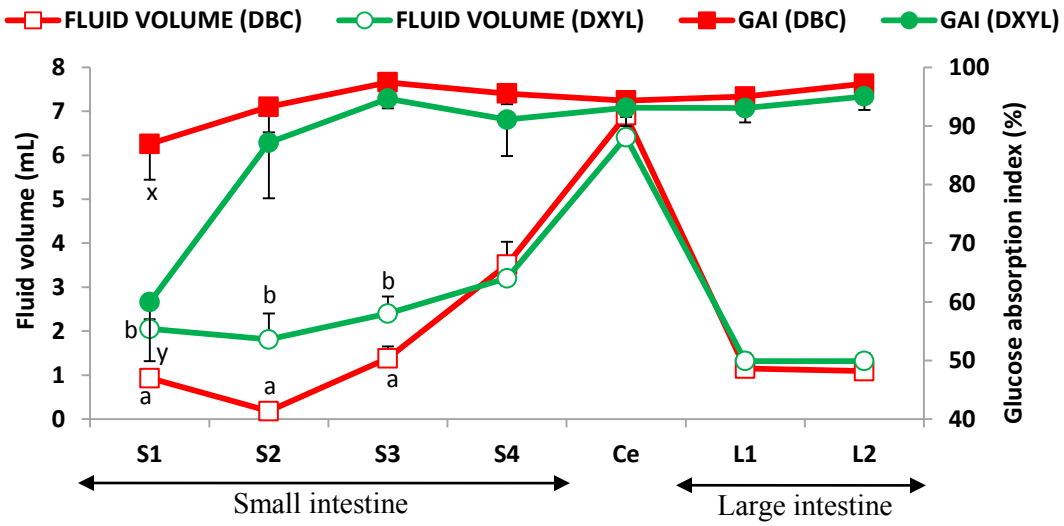
Data are presented as mean ± SD of five to six animals. Different letters presented in each column for a given segment are significantly different from each other group of animals ($p < 0.05$. Tukey's HSD post-hoc test, IBM, SPSS, version 21).

The data for percentage of digesta transit in the different segments of the GIT of all groups are presented in Table 4.1. The digesta transit for all groups was generally decreased from the small intestine to the cecum, and increased from the cecum to the colon. Although the digesta transit in the 1st and 2nd quarters of the small intestine did not differ significantly when comparing between the groups, the digesta transit of the xylitol fed groups (NXYL and DXYL) was relatively higher than their respective controls groups (NC and DBC). Nevertheless, in the 3rd quarter of the small intestine, the digesta transit of the NXYL and DXYL groups was significantly higher ($p< 0.05$) compared to the NC, DBC and DBA groups, when there was no significant difference between the NC, DBC and DBA groups, as well as between the NXYL and DXYL groups (Table 4.1). There was no significant difference between all groups in the last quarter of the small intestine. However, in the cecum, the digesta transit of the NXYL and DXYL groups was significantly lower ($p< 0.05$) compared to the NC and DBA groups but relatively lower compared to the DBC group, when no significant different was observed between the NC, DBC and DBA groups, as

well as between the NXYL and DXYL groups. Digeta transit was not significantly affected at all by xylitol ingestion in either normal or diabetic animals in the proximal half of the colon.



(a)

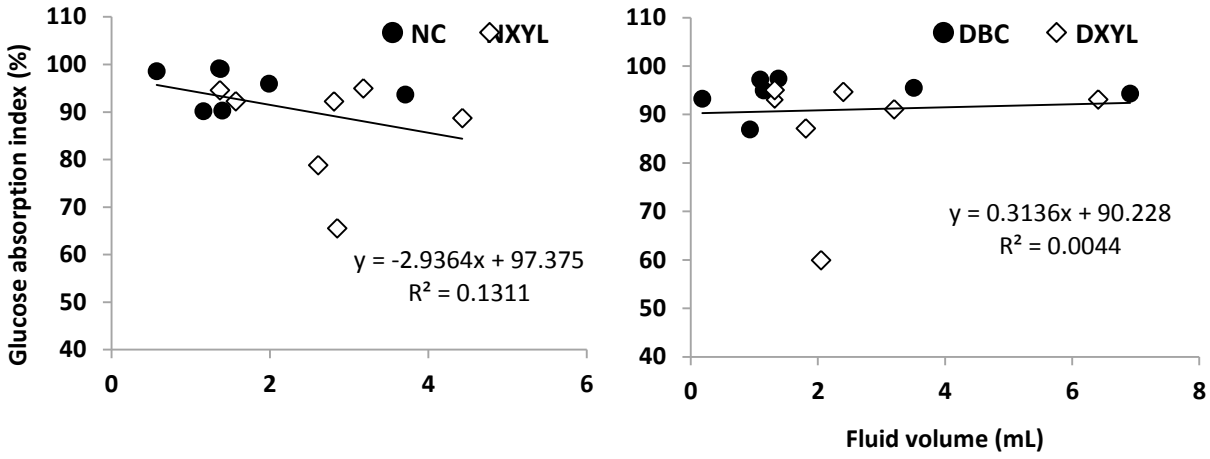


(b)

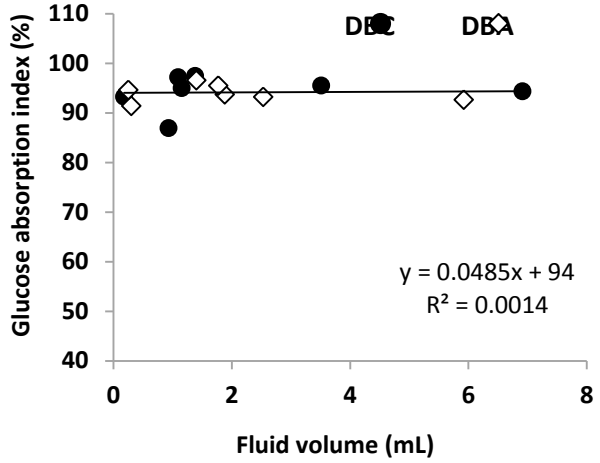
Figure 4.7: Data showing the relationship between the intestinal fluid volume and glucose absorption index in the different segments of the GIT of NC and NXYL groups **(a)** and DBC and DXYL groups **(b)**. *Data are presented as mean ± SD of five to six animals.Different letters ("a" and "b"or "x" and "y") presented near the lines for a given segment of each parameter (fluid volume or GAI respectively) are significantly different from each other ($p < 0.05$. Tukey's HSD post-hoc test, IBM, SPSS, version 21).*

The data showing the relationship between the fluid volume and glucose absorption index (GAI) are presented in Figure 4.7. The fluid volume of all groups, which was equivalent to the mass of content in each segment of the GIT (i.e., 1 g of content was considered equivalent to 1 mL of fluid volume) was highest in the cecum compared to other segments of the GIT (Figure 4.7a and b). In the 1st, 2nd and 3rd quarters of the small intestine, the fluid volume of the xylitol treated groups (NXYL and DXYL) was significantly higher ($p< 0.05$) compared to their corresponding control groups (NC and DBC) (Figure 4.7a and b). In the last quarter of the small intestine, the fluid volume of the NXYL group was significantly higher ($p< 0.05$) than the NC (Figure 4.7a), when there was no significant difference between the DBC and DXYL groups (Figure 4.7b). In the proximal and distal halves of the colon, the fluid volume of the NXYL group was significantly higher and lower ($p< 0.05$) respectively compared to the NC group (Figure 4.7a), when no significant difference was observed between the DBC and DXYL groups of similar segments (Figure 4.7b). In the cecum, no significant difference was observed between the NC and NXYL groups and between the DBC and DXYL groups (Figure 4.7a and b). The overall relation between the fluid volume and GAI in the GIT was an inverse relation, i.e. the more the fluid volume, the lesser the GAI at the different segments of the GIT (Figure 4.7a and b).

The correlation graphs between fluid volume and GAI in the different segments ofthe GIT are presented in Figure 4.8, which showed that both parameters across all segments of the GIT in the non-diabetic groups were inversely related or proportional, but no significant correlation was observed ($p= 0.203$) between the fluid volume and GAI in the entire GIT (Figure 4.8a).



(a): NC and NXYL(b): DBC and DXYL



(c): DBC and DBA

Figure 4.8: Correlation plot between the glucose absorption index (Y axis) and intestinal fluid volume (X axis) in the different segments of theGIT of NC-and NXYL-**(a)**; DBC- and DXYL-**(b)**; DBC-and DBA- **(c)**.*Data are presented as the mean ± SD of five to six animals. Significant correlation denotes p < 0.05 (Analysis ToolPak, Microsoft excel, Microsoft Corporation).*

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

In recent years, xylitol is being widely used as a sugar substitute because of its severalbeneficial effects on health compared to other commonly used sweeteners. The lower caloric value (2.4 kcal vs 4.0 kcal/g), insulinemic response, lower glycemic index (13) and similar sweetness compared to sucrosehave made it more popular to poeple. In a recent study, it has been reported that xylitol exhibits significant anti-diabetic and hypoglycemic effects in a type 2 diabetes model of rats(Islam and Indarjit, 2012). The present study was conducted in order to investigate and understand the possible mechanisms behind the anti-diabetic effects of xylitol using several *in vitro* and *in vivo* models.

The action of carbohydrate hydrolyzing enzymes on carbohydrate-containing diet has a significant effect on postprandial blood glucose level, and delaying the digestion of carbohydrate like starch and sucrose will translate into lower postprandial blood glucose (Dhital *et al.,* 2013; Reuser and Wisselaaar, 1994). Alpha glucosidase and alpha amylase inhibitors have gained much popularity as a class of hypoglycemic agents, which reduces postprandial blood glucose via this mechanism by inhibiting the action of alpha glucosidase and alpha amylase enzymes (Bischoff, 1994 and 1995; Patel *et al.,* 2012). The result from our *in vitro* study showed a significant inhibition of alpha amylase and alpha glucosidase enzyme activities by xylitol (Figure 4.1a and b), which is an indication that xylitol may possess significant inhibitory effect on carbohydrate breakdown. This can result in reducing the levels of postprandial blood glucose, which may partly be involved in the mechanism behind the hypoglycemic effects of xylitol.

The major dietary source of glucose for human comes from starchy foods and other similar carbohydrates, and the degree of absorption of digested starch (glucose) is an important factor that can play a major role in the development of diabetes, obesity and related metabolic disorders (Dhital *et al*., 2013). Glucose is rapidly absorbed in the intestine by a mechanism of facilitated diffusion, and results from previous *in vitro* studies have suggested the elevated capacity of glucose absorption across all segments of the small intestine (Fearson and Bird, 1968), with the mid-small intestine (part of the duodenum and jejunum) having the highest absorption capacity (Lavin, 1976). In the present study, a 2 hour incubation of isolated rat jejunum in a glucose solution with the villi exposed, showed glucose absorption as high as 3.27 ± 0.46 mg/cm of jejunum in control or when no xylitol was used. However, in the presence of 40% xylitol, there

was a significant reduction in the glucose absorption capacity (1.75 ± 0.46 mg/cm of jejunum), which is an indication of the possible inhibitory potentials of xylitol on intestinal glucose absorption, which might be partly involved in the mechanism behind the hypoglycemic effects of xylitol.

Among other functions, insulin produced in the body helps to stimulate the uptake of circulating glucose by actively respiratory cells for energy production, thus lowering and maintaining blood glucose homeostasis (Mathews *et al*., 2000). In previous study, Muller-Hess *et al*., (1975) reported that blood glucose and serum insulin was significantly increased by oral administration of 30 or 50 g of xylitol in normal subjects, while in some other studies, it has been reported that 3 to 5 weeks oral administration of 10% xylitol increased serum insulin in both normal and diabetic rats (Islam, 2011; Islam and Indarjit, 2012). Since the reduction in circulating glucose uptake can cause reduced clearance of blood glucose, which could be a pathogenic route to the observed hyperglycemia in diabetic rats (Chaiken *et al*., 1993), it is therefore rationale and reasonable to investigate the effects of xylitol on glucose uptake in isolated rat psoas muscle using an *in vitro* model. The results from our study showed that xylitol significantly increased glucose uptake in isolated rat psoas muscle compared to the control (without xylitol). This suggests that xylitol may improve the uptake of circulating glucose, which might partly contribute to the anti-diabetic as well as hypoglycemic potential of xylitol. Insulin did not significantly contribute to the glucose uptake of xylitol, although it appreciably increased glucose uptake in the control and 10% xylitol. However, direct effect of xylitol on muscle glucose uptake without insulin suggests thatxylitol may have insulin-like effects on skeletal muscle in the stimulation of circulating glucose uptake (Abdel-Sattar *et al*., 2012; Gupta *et al*., 2005).

The concentration range of xylitol used in the *ex vivo*studies were chosen based on the concentration of xylitol used in previous study (Islam, 2011; Islam and Indarjit, 2012). Although these concentrations were high relative to the concentration of glucose used, the results suggested concentration dependent effects of xylitol, comparing the effects of different xylitol concentrations.

In order to further investigate the inhibitory potential of xylitol on intestinal glucose absorption in *in vitro* environment, an *in vivo* study was conducted and several intestinal nutrient

absorption-related parameters that can directly or indirectly affect the glucose absorption were estimated. Phenol red was used as a recovery marker in this *in vivo* study (Islam and Sakaguchi, 2006).

Unlike most polyols, glucose is rapidly and completely absorbed in the small intestine. Several *in vivo* studies have reported the different absorption pattern of glucose in the small intestine. Bogner *et al*., (1963), reported that glucose absorption was highest in the mid-intestine of female chicks, when Lavin(1976) suggested the ileum as a highest glucose absorbing section in the small intestine of chicks. On the other hand Riesenfeld *et al*., (1980) reported a reduction in glucose absorption capacity with increasing distance from the pylorous, and later explained that the difference in glucose absorption capacity in the different segments of the small intestine may be due to variation of concentrations. However, results of our present study did not show much difference in glucose absorption index (GAI) between the different segments of the small intestine, which corresponds with findings reported by Fearson and Bird(1968). However, the significantly reduced GAI in the xylitol fed groups (NXYL and DXYL) compared to their corresponding controls (NC and DBC) across the small intestinal segments (more pronounced in the 1st and 2nd quarters)correspond with the results of our*in vitro*glucose absorption studies, and supports the inhibitory potentials of xylitol on intestinal glucose absorption. This may be partly involved in the mechanism behind the hypoglycemic as well as anti-diabetic effects of xylitol in normal or diabetic conditions.

Furthermore, in a recent study, Hassan *et al*., (2010) reported that 3.0 mM acarbose caused significant decrease in glucose intestinal absorption *in vitro*. However in our present study, acarbose did not significantly affect intestinal glucose absorption both*in vitro* and *in vivo* environment, which could be due to its mode of action. Acarbose is a known alpha amylase and alpha glucosidase inhibitor that inhibits the breakdown of glucose-building carbohydrates like starch and maltose, hence delaying the release of glucose from carbohydrate (Bischoff, 1994; Patel *et al*., 2012). However, in our study, glucose was used either as a substrate or administered orally; hence acarbose did not exert any significant effect on intestinal glucose absorption.

The rate of nutrient gastric emptying and digesta transit are important factors in gastro intestinal nutrient digestion and absorption (Islam and Indarjit, 2012). It has also been reported that faster intestinal transit and delayed gastric emptying might be the cause of slow intestinal nutrient

absorption and reduced food intake (Salminen *et al*., 1984; Shafer *et al*., 1987). In our present study, although not significantly, induction of diabetes appreciably increased the rate of nutrient gastric emptying, which correspond with the lower phenol distribution in DBC group compared to the NC group (Figure 4.4 and 4.5).Although negative energy balance resulting from constant hyperglycemiais known as a major reason for the frequent hunger (polyphagia) and increased food intake often observed as a classical symptom of diabetes, faster gastric emptying may also contribute to this effect. Previous studies have demonstrated a correlation between gastric emptying rate and eating rate or satiety (Bergmann *et al*., 1992; Zhu *et al*., 2013). However, the oral administration of a single bolus dose of xylitol delayed gastric emptying after 1 hour in both normal and diabetic rats, which correlates with the higher phenol red distribution in the stomach of the xylitol fed rats (Figure 4.4 and 4.5). This trend corresponds to studies previously reported by Shafer *et al*., (1987), and may also be the cause of the reduced food intake in diabetic rats fed with xylitol as reported in a very recent study(Islam and Indarjit, 2012).

Furthermore, despite the delayed gastric emptying caused by a single dose of xylitol administration, it also caused a faster digesta transit from the small intestine towards the cecum. This, coupled with a concomitant delay of gastric emptying by xylitol, corresponds to data published by Salminen *et al*., (1989) in normal human subjects, and may also contribute to the reduced GAI observed in the small intestine of the xylitol treated groups in this study. The relatively smaller amount of ingested nutrient or glucose reaching the small intestine from the stomach in the xylitol fed groups, as indicated by the slower gastric emptying, and a concomitant faster transit of small intestinal nutrient can cause reduced glucose absorption, which may partly contribute to the mechanism behind the anti-diabetic as well as hypoglycemic effect of xylitol.

Also, the volume and transit of intestinal fluid are factors that can play an important role in determining the extent of small intestinal nutrient digestion and absorption (Islam and Sakaguchi, 2006). A correlation graph of GAI (%) versus fluid volume (mL) showed an inverse, but insignificant correlation($p< 0.05$) between both parameters (figure 4.8a). In other words, where there was lower glucose absorption in the intestine, there was a corresponding higher fluid volume in that particular segment of the small intestine. Although, from the correlation plot, not all groups satisfied this relationship between absorption and fluid volume (figure 8.4b and c), the graph on figure 4.7a and b suggests higher glucose absorption when there was a lower fluid

volume in the different segments of the intestine for most groups. Furthermore,Islam and Sakaguchi (2006) reported that high intestinal nutrient transit coupled with excessively high intestinal fluid volume have the possibility of reducing degradation and absorption by causing lesser amounts of intestinal content to come into contact with the intestinal mucosal surface. In our present study, the higher intestinal fluid volume and faster nutrient transit in the small intestine of the xylitol fed groups may have also contributed to the concomitantly lower glucose absorption in the small intestine of these groups.

5.2 Conclusion

The results from this study suggest that xylitol has inhibitory effects on the activities of carbohydrate hydrolyzing enzymes; alpha amylase and alpha glucosidase *in vitro*, and delay effects onsmall intestinal glucose absorption, especially in the duodenal and jejunal segments *in vitro* and *in vivo*. The results also suggest that xylitol can prolong gastric emptying and increase the rate of intestinal nutrient transit *in vivo* in both normal and diabetic conditions, which may partly contribute to its inhibitory potential on intestinal glucose absorption. Furthermore, results of this study also suggest that xylitol may also promote the uptake of circulating glucose by muscle tissue, which has been proven by our *in vitro* muscle glucose uptake study.

The above mentioned hypoglycemic potentials of xylitol, especially in the *in vitro* study, were dose dependent. These effects were shown to be most significant at 30% and 40% concentrations of xylitol, which are below the threshold range that can elicit gastrointestinal discomfort in adults and children (Bond and Dunning, 2006; Makinen, 1976;Wang and van Eys, 1981). Moreover, it has been reported that regular ingestion of xylitol increases tolerance against gastrointestinal discomfort caused by xylitol (Wang and van Eys, 1981), and it is also a safer sugar alcohol compared to many other available in the market (Islam, 2011).

In conclusion, the anti-diabetic effects of xylitol may not only be due to inhibition of carbohydrate digestion and glucose absorption from the small intestinal mucosa; but may also be due to improving insulin action or exhibiting an insulin-like effect on skeletal muscle, thus increasing muscle glucose uptake and utilization. This may also be mimicked under *in vivo* conditions.

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