Studies on the Anti-hyperglycemic Potentials and Possible Mode of Actions of Some Commonly Used Sugar Alcohols

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Submitted in fulfillment of the academic requirements for the degree of Doctor of Philosophy in Biochemistry, School of Life Sciences, University of KwaZulu-Natal (Westville campus), Durban 4000, South Africa

SUPERVISOR: Prof. M. S. ISLAM (Ph. D.)

PREFACE

The information presented in this thesis is an original work by the candidate. It was carried out in the Department of Biochemistry, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, Durban, South Africa from February, 2014 to December, 2016 under the supervision of Prof. M. S. Islam and has not otherwise been submitted in any form for any degree or diploma to any other University. Where use has been made of the work of others, it has been duly acknowledged in the text in the form of reference.

Candidate: Chika Ifeanyi Chukwuma

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DECLARATION 1 - PLAGIARISM

I, **Chika Ifeanyi Chukwuma**, 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.

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   a.   Their words have been re-written but the general information attributed to them has been referenced

   b.   Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

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.

Signed

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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 Chika I. Chukwuma under the supervision of Prof. M.S. Islam (PhD), while the editorial work was done by Prof. M.S. Islam (PhD).

PUBLICATIONS FROM THIS THESIS

1. **Chukwuma CI**, Ibrahim MA, Islam MS (2016) Maltitol inhibits small intestinal glucose absorption and increases insulin mediated muscle glucose uptake *ex vivo* but not in normal and type 2 diabetic rats. **International Journal of Food Science and Nutrition**, 12: 1-9.

2. **Chukwuma CI**, Ibrahim MA, Islam MS (2016) Myo-inositol inhibits intestinal glucose absorption and promotes muscle glucose uptake: a dual approach study. **Journal of Physiology and Biochemistry**, 72(4): 791-801.

3. **Chukwuma CI**, Islam MS (2016) Xylitol: one name, numerous benefits. In: Mérillon JM, Ramawat KG (eds) Sweeteners, **Reference Series in Phytochemistry, Springer International Publishing Switzerland**, doi: 10.1007/978-3-319-26478-3_33-1.

4. **Chukwuma CI**, Islam MS (2016) Sorbitol increases muscle glucose uptake *ex vivo* and inhibits intestinal glucose absorption *ex vivo* and in normal and type 2 diabetic rats. **Applied Physiology, Nutrition and Metabolism [RCS Press, Canada]**, doi: 10.1139/apnm-2016-0433.

5. **Chukwuma CI**, Islam MS. Erythritol reduces small intestinal glucose absorption, increases muscle glucose uptake, improves glucose metabolic enzymes activities and increases mRNA expression of glut-4 and irs-1 in type 2 diabetic rats (**submitted to Molecular Nutrition and Food Research; under revision for potential acceptance**).

OTHER PUBLICATIONS

6. Erukainure OL, Mesaik AM, Muhammad A, **Chukwuma CI**, Manhas N, Singh P, Aremu OS, Islam MS (2016) Flowers of *Clerodendrum volubile* exacerbate immunomodulation by suppressing phagocytic oxidative burst and modulation of COX-2 activity. **Biomedicine & Pharmacotherapy**, 83: 1478–1484.

7. **Chukwuma CI**, Islam MS. Xylitol improves anti-oxidative defense system in serum, liver, heart, kidney and pancreas of normal and type 2 diabetes model of rats. Accepted in **Acta Poloniae Pharmaceutica**, **2016**.

8. **Chukwuma CI**, Islam MS (2015) Effects of xylitol on carbohydrate digesting enzymes activity, intestinal glucose absorption and muscle glucose uptake: a multi-mode study. **Food and Function**, 6: 955-62.

PRESENTATIONS

1. **Chukwuma CI**, Islam MS. Studies on the anti-hyperglycemic potential of sorbitol and possible mechanisms of action. **School of Life Science Post-graduate Research Day**, 20 May, 2016 at University of KwaZulu-Natal, Golf Rd Campus, Pietermaritzburg 3221, South Africa. (**Oral presentation**)

2. **Chukwuma CI**, Islam MS. Effect of xylitol on the anti-oxidative defense system in a type 2 diabetes model of rats. **The 23rd IDF World Diabetes Congress**, 30 November – 4 December, 2015 at Vancouver Convention and Exhibition Center, Vancouver, Canada. (**Poster presentation**)

3. **Chukwuma CI**, Islam MS. Effects of Sorbitol on carbohydrate digesting enzymes activity, intestinal glucose absorption and muscle glucose uptake: A multi-mode study. **College of Agriculture, Engineering and Science Post-graduate Research Day**, 22 September, 2015 at the Commerce Block (C Block), University of KwaZulu-Natal, Golf Rd Campus, Pietermaritzburg 3221, South Africa. (**Oral presentation**). *[I was awarded 2nd prize as one of the Best oral presenters from the School of Life Sciences]*

4. **Chukwuma CI**, Islam MS. Xylitol reduces intestinal glucose absorption via inhibiting major carbohydrate digesting enzymes, slowing gastric emptying and fastening intestinal

transit rate but increases muscle glucose uptake in normal and type 2 diabetic rats: a multi-mode study. **College of Agriculture, Engineering and Science Post-graduate Research Day**, 27 October, 2014 at T Block Building, University of KwaZulu-Natal, Westville Campus, Durban 4000, South Africa (**Oral presentation**).*[I was awarded 1st prize as one of the Best oral presenters from the School of Life Sciences]*

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

Ace-k	Acesulfame potassium
ADA	American Diabetes Association
ADI	Accepted daily intake
AGEs	Advanced glycation end products
C_T	Cycle threshold
CVDs	Cardiovascular disorders
DBA	Diabetic acarbose
DBC	Diabetic control
DEPC	Diethylpyrocarbonate
DER	Diabetic erythritol
DMI	Diabetic myo-inositol
DML	Diabetic maltitol
DPP-IV	Dipeptidyl peptidase IV
DSO	Diabetic sorbitol
E	qPCR efficiency
FBG	Fasting blood glucose
FDA	Food and Drug Administration
GAI	Glucose absorption index
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide 1
Glut-4	Glucose transporter type 4
HbA1c	Glycated hemoglobin
IDF	International Diabetes Federation
IRS-1	Insulin receptor substrate 1 family
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MOPS	3-(N-morpholino) propanesulfonic acid
NC	Normal control
NEFA	Non-esterified fatty acid
NER	Normal erythritol
NFBG	Non-fasting blood glucose

NIDDM	Non-insulindependent diabetes mellitus
NMI	Normal myo-inositol
NML	Normal maltitol
NNS	Non-nutritive sweetener-
NSO	Normal sorbitol
OGTT	Oral glucose tolerance test
PCOS	polycystic ovary syndrome
PI3-K	Phosphoinositide 3-kinase
PPAR-γ	Peroxisomes proliferator-activated gamma
PR	Phenol red
qPCR	Real-Time PCR
RGR	Relative glycemic response
RIR	Relative insulin response
SD	Sprague-Dawley
SEMDSA	Society for Endocrinology, Metabolism and Diabetes of South Africa
T1D	Type 1 diabetes
T2D	Type 2 diabetes
VEGF	Vascular endothelial growth factor

ABSTRACT OF THESIS

The present study investigated the effects of four commonly used sugar alcohols (maltitol, myo-inositol, sorbitol and erythritol) on intestinal glucose absorption and muscle glucose uptake as possible anti-hyperglycemic agents and their mechanisms of action using some *ex vivo* and *in vivo* experimental models.

Data of the *ex vivo* study (experiment 1) showed that all sugar alcohol exhibited a concentration-dependent (2.5 – 20%) inhibitory effect on jejunal glucose absorption and enhancement of psoas muscle glucose uptake (with or without insulin) *ex vivo*. Maltitol showed the most potent inhibitory effect on intestinal glucose absorption ($IC_{50} = 3.57 \pm 1.18\%$) but poor muscle glucose uptake effect without insulin ($GU_{50} = 111.12 \pm 19.36$) *ex vivo*. On the other hand, erythritol showed the most potent muscle glucose uptake effect with ($GU_{50} = 2.02 \pm 1.24\%$) or without insulin ($GU_{50} = 6.91 \pm 2.03\%$), but its inhibitory effect on intestinal glucose absorption *ex vivo* was not remarkable ($IC_{50} = 55.90 \pm 11.98\%$). Data of the *in vivo* glucose absorption study (experiment 2) revealed that excluding maltitol, a bolus tolerable dose of sorbitol (0.4 g/kg bw), myo-inositol (1 g/kg bw) and erythritol (1 g/kg bw) co-ingested with glucose (2 g/kg bw) significantly ($p < 0.05$) delayed gastric emptying, accelerated digesta transit and significantly ($p < 0.05$) reduced postprandial blood glucose increase as well as glucose absorption at the proximal quarter of the small intestine of normal and type 2 diabetic rats. Further *in vivo* glucose uptake study (experiment 3) arising from the outstanding *ex vivo* muscle glucose uptake potentials of erythritol revealed that a single bolus dose of erythritol (1 g/kg bw) appreciably improved glucose tolerance, insulin secretion, mRNA expression of muscle Glut-4 and IRS-1 as well as the activities of muscle and liver hexokinase, but suppressed the hepatic release of glucose in diabetic animals.

Data of this study suggest that myo-inositol, sorbitol and erythritol, but not maltitol exhibited anti-hyperglycemic potentials, possibly via inhibiting intestinal glucose absorption and enhancing muscle glucose uptake. Hence, these agents may be further investigated to access their anti-hyperglycemic potentials to be used not only as sweeteners but also as supplements to the food and food products of type 2 diabetics. However, further clinical studies are warranted in this regard.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1   General introduction and background

Diabetes mellitus is a metabolic disorder caused by a defect in insulin secretion, insulin action, or both (Bastaki, 2005). Recent data from the International Diabetes Federation (IDF) reported that the global estimate of people with diabetes is about 415 million and this figure has been projected to become 642 million by 2040 (IDF, 2015). There are several types of diabetes, but among the two major types (type 1 and type 2), type 2 is more prevalent, accounting for more than 90% of all diabetes cases (Loghmani, 2005; Pratley, 2013). Type 2 diabetes (T2D) is described as a multifaceted and progressive disease that is linked to several metabolic defects and organ malfunction or damage (Pratley, 2013). The most prominent metabolic defects contributing to the pathogenesis and progression of T2D are impaired insulin secretion and insulin resistance in peripheral tissues, such as adipose, muscle and hepatic tissues (Pratley &Weyer, 2001; Kahn, 2003). Impaired insulin secretion is due to a progressive loss of pancreatic beta cell function and beta cell mass, which has been observed in the onset (pre-diabetes) and progression of T2D (Pratley & Weyer, 2001; Kahn, 2003).

Additionally, the interaction between several factors such as genetic, environmental and behavioural risk factors is strongly involved in the pathogenesis and progression of T2D (Olokoba *et al*., 2012; Wu *et al*., 2014). Behavioural risk factors are the most influential, which includes sedentary life style, routine consumption of high fat and high calorie diets as well as chronic consumption of refined and simple carbohydrates e.g. sucrose, fructose etc (Uusitupa, 2002; Hu, 2003; Montonen *et al*., 2007). Several studies have consistently reported the role of sugar consumption in the development of T2D and metabolic syndrome (Elliott*et al*., 2002; Astrup *et al*., 2002; Hu & Malik, 2010; Stanhope, 2012). Accordingly, the use of sugar substitutes and non-nutritive sweeteners as part of diabetic and non-diabetic diets is increasingly gaining public endorsement in the management of diabetes, because they have minimal effect in increasing post-prandial blood glucose and caloric value compared to easily digestible simple carbohydrates such as glucose, sucrose and fructose (Talbot & Fisher, 1978; Brown *et al*., 2010).

Sugar substitutes are known for their sweetening ability and are usually used to replace regular table sugar in numerous edible products (Islam, 2011). They have negligible or reduced effect on blood glucose and insulin levels and also provide lower calorie compared to glucose and sucrose (Islam, 2011). Non-nutritive or artificial sweeteners like saccharin, aspartame, sucralose, neotame, ace-K etc are some of the sugar substitutes that are consumed by humans. They are characterized by an intense sweetening power ranging from 30-13000 times as well as a zero glycemic, insulinenic and caloric value compared to sucrose (O'Donnell & Kearsley, 2012; Fitch & Keim, 2012).

Although non-nutritive sweeteners (NNS) are widely used in diabetic and non-diabetic diets with determined accepted daily intake (ADI), controversies regarding their safety has been documented (Tandel, 2011). This is because of some life threatening side effects such as carcinogenic or cancerous effects, migraines, genetic diseases, etc that may be associated with the chronic consumption of some of these NNS (Islam & Indarjit, 2012). Additionally, most of the time, it is not feasible to adhere to their determined ADIs, since NNS are used in many products consumed by people regularly. On the other hand, nutritive or reduced calorie sweeteners like sugar alcohols are non-synthetic and do not pose any life threatening side effects if consumed at tolerable dosages. The commonly used sugar alcohols are xylitol, erythritol, maltitol, isomalt, mannitol, myo-inositol, lactitol and sorbitol, which have a number of beneficial effects on health including dental protection (Van Loveren, 2004). They have lower caloric effect and glycemic response compared to glucose and sucrose (Livesey, 2003). They are used as sweeteners and additives in numerous dental and oral health products. Nevertheless, the beneficial effects of sugar alcohols are not limited to the above stated benefits. Data from several studies suggest that sugar alcohols may also possess anti-hyperglycemic potentials in normal and diabetic conditions (Ishikawa *et al*., 1996; Natah *et al*., 1997; Maeba *et al*., 2008; Islam and Indrajit, 2012; Kang *et al.,* 2014; Chukwuma and Islam, 2015; Woelnerhanssen *et al*., 2016), which have not been completely elucidated so far.

In a previous study, it has been reported that 14 days daily consumption of 20 g of erythritol significantly reduced blood glycated hemoglobin (HbA1c); relatively reduced blood glucose levels; and had no significant effects on serum lipids and renal function in subjects with non-insulin dependent diabetes mellitus (NIDDM) (Ishikawa *et al*., 1996). In other studies,

mannitol and lactitol has been reported to reduce hyperglycemia in healthy human subjects (Mäkinen and Hämäläinen, 1985; Natah *et al*., 1997). Furthermore, dietary myo-inositol significantly decreased blood glucose (Maeba *et al*., 2008) and also improved insulin sensitivity biomarkers in human subjects with metabolic syndrome (Giordano *et al*., 2011; Santamaria *et al*., 2012). Additionally, Moon *et al*. (1990) reported that dietary maltitol significantly reduced glycemic and insulin response compared to glucose in normoglycemic and diabetic subjects (Moon *et al*., 1990). In a more recent study, the *in vitro* α-glucosidase, α-amylase and sucrase inhibitory activities of some commonly used sugar alcohols was reported (Kang *et al.,* 2014). TheJoint FAO/WHO Expert Committee on Food Additives (JECFA) has proposed that sorbitol may possess inhibitory effects on glucose absorption based on data from several studies (World Health Organization, 1985). In a very recent study, Chukwuma and Islam (2015) demonstrated the potency of xylitol on the inhibition of intestinal glucose absorption and enhancement of insulin-mediated muscle glucose uptake *ex vivo* and in normal and type 2 diabetic rats (Chukwuma and Islam, 2015).

Despite the above-stated anti-hyperglycemic potentials of some commonly used sugar alcohols, further investigations are still required for most of them to elucidate their additional anti-hyperglycemic potency and the mechanisms of action. Therefore, the present study was conducted to investigate the effects of some selected and commonly used sugar alcohols on intestinal glucose absorption and muscle glucose uptake as a possible anti-hyperglycemic mechanism of action.

1.2   Literature review

1.2.1   Diabetes

Diabetes has been a plague to man for more than 3000 years, which was initially characterized by the sweetness of the urine and blood of patients, first observed in ancient Indians (Ahmed, 2002). As time progressed, the role of glucose metabolism and pancreas malfunction in the pathogenesis of diabetes was established (Ahmed, 2002; Piero *et al*., 2014). Presently, diabetes is defined as a multifaceted or heterogenous disorder that is peculiarly associated with abnormally high blood glucose due to endocrine and/or metabolic disorders (Lin & Sun, 2010). These

disorders lead to blurred vision, frequent hunger (polyphagia), weight loss, excessive urination (polyuria), increased thirst (polydipsia) and a compensatory increased fluid intake, which are the other clinical symptoms of diabetes other than hyperglycemia (Lin & Sun, 2010). Diabetes has been classified into several types based on the aetiology and clinical presentation.

1.2.2 Types of diabetes

There are several types of diabetes, which are classified based on their pathogenic processes. According to the American Diabetes Association (ADA), these include the auto-immune destruction of pancreatic beta cells, deficient insulin production or secretion and resistance of peripheral tissues to insulin action (ADA, 2004). These abnormalities may exist separately or co-exists in patients having different diabetic conditions. The major types of diabetes are type 1 diabetes (T1D), T2D and gestational diabetes. Other forms of diabetes-related endocrine or metabolic defects are closely associated with the pathophysiology of T1D and T2D.

i) Type 1 diabetes or insulin-dependent diabetes mellitus

This form of diabetes was formally referred to as insulin-dependent diabetes mellitus. It is caused by auto-immune destruction of the insulin-producing pancreatic beta cell, which causes insulin deficiency due to inadequate secretion of insulin by beta-cells (Atkinson & Eisenbarth, 2001). Accordingly, patients with T1D may require exogenous insulin to compensate for depleted insulin secretion. Studies have shown that islet destruction by auto-antibodies is common among infants, who show early characteristic of islet auto-immunity as early as before the age of 2 years (Ziegler *et al*., 1999; Kimpimaki *et al*., 2002; Hummel *et al*., 2004). Hence, the name "juvenile-onset diabetes" was used to describe this form of diabetes. Several factors influence the development of T1D, including genetic, environmental and dietary factors, viral infections and transmission of islet auto-antibodies from mother to offspring and so on (Achenbach, 2005).

ii)   Type 2 diabetes or non-insulindependent diabetes mellitus

In the onset of T2D, peripheral tissues do not effectively respond to the insulin action in clearing of circulating blood glucose and pancreatic beta cells do not adequately secrete insulin hormone. Over time, type 2 diabetic patients experience persistent hyperglycemia due to partial pancreatic beta-cell dysfunction, impaired glucose tolerance and insulin resistance (Pratley & Weyer, 2001; Kahn, 2003). The inability of peripheral tissues to respond to insulin action is known as insulin resistance.

iii)   Gestational diabetes

This is a type of hyperglycemia that is common to pregnant women, which is caused by glucose intolerance during the second to third trimester of their pregnancy (Buchanan and Xiang, 2005). Pregnancy causes a state of insulin resistance and hyperinsulinemia that may put pregnant women at risk of developing gestational diabetes (Buchanan &Xiang, 2005; Gilmartin *et al*., 2008). Insulin resistance during pregnancy may be due to several reasons, including growth hormone alteration; secretion of cortisol, an insulin antagonist; increase in calorie intake and maternal adipose deposition; lactogen and insulinase secretion in the placenta (Gilmartin *et al*., 2008). Between 2% to 5% of pregnant women encounter gestational diabetes, but varies according to the prevalence of type 2 diabetes (Xiong *et al*., 2001; Gilmartin *et al*., 2008). Risk factors of gestational diabetes include history of macrosomia (birth weight > 4000 g), polycystic ovarian syndrome, obesity, hypertension, persistent glucosuria, spontaneous abortions as well as a family history of diabetes (Xiong *et al*., 2001; Gilmartin *et al*., 2008). Supporting data have shown a correlation between child birth weight above the 90th percentile and increasing plasma glucose levels (HAPO study, 2008).

iv)   Other specific diabetes-related defects and causes

There are other types of diabetes-related defects that could be associated with hyperglycemia. Some forms of diabetes are linked to a genetic defect of genes (e.g. glucokinase gene) and gene transcription factors (e.g. hepatocyte nuclear factor (HNF)-1α, HNF-4α, HNF-1β and insulin

promoter factor (IPF)-1), which are involved in glucose metabolism (ADA, 2004). These defected genes or transcription factors can be inherited in an autosomal dominant pattern. They usually occur at the onset of maturity in patients (mostly before 25 years of age). A typical example is the defect that is associated with mutation on chromosome 7p of the glucokinase gene, which causes an altered insulin secretion and eventually, hyperglycemia (ADA, 2004). Another example is the mutation of insulin receptor genes, which adversely affect insulin action on peripheral tissues as well as cellular uptake of circulating glucose.

Furthermore, pancreatic injuries and infections such as pancreatitis, trauma, viral infection, pancreatectomy and pancreatic carcinoma can damage the pancreas and eventually cause diabetes due to insufficient insulin secretion to regulate blood glucose. Additionally, exogenous substances such as drugs and chemicals may also induce diabetes by either impairing insulin secretion and action or acting as a toxin that can permanently damage pancreatic beta-cell (Blackburn & Wilson, 2006; Rehman *et al*., 2011; Tripathi & Verma, 2014). In some other instances, diabetes can be caused by genetic in-born error syndromes. For example, in Wolfram's syndrome there is an autosomal recessive disorder that is linked to insulin-deficiency and absence of β-cells at autopsy, which causes diabetes insipidus (ADA, 2004). Finally, some hormones e.g. growth hormone, cortisol, glucagon, and epinephrine are known to be insulin antagonists, because they can antagonize the action of insulin (Lager, 1991) and consequently predispose victims to hyperglycemia.

1.2.3   Prevalence of diabetes

Diabetes is a serious threat to global public health, which has become a major cause of morbidity and mortality around the world including in the developed countries (Menke *et al*., 2015). The prevalence of this disease is tremendously increasing with population growth due to aging, urbanization, unhealthy eating habit, sedentary life style and increasing prevalence of obesity (Wild *et al*., 2004). About 171 million people of all age group worldwide (approximately 2.8%) were diagnosed to have diabetes in the year 2000, and this figure has been predicted to increase to about 336 million people (approximately 4.4%) by the year 2030 (Wild *et al*., 2004). Demographic studies suggest that this increase is mainly in the proportion of people > 65 years

of age. As at 2015, the global figure of people living with diabetes between age 20 and 79 years was already about 415 million (meaning approximately 1 in every 11 adult is diabetic) (IDF, 2015). The new projection of diabetes by the year 2040 is approximately 642 million (meaning approximately 1 in every 10 adult will be diabetic) (**Table 1.1**) (IDF, 2015). Additionally, the global health expenditure to treat diabetes and prevent complications was more than 600 billion USD in the year 2015, making diabetes a significant opposition to economic development and sustainable growth of healthcare systems (IDF, 2015).

Table 1.1: Global percentage of people (20-79 years) living with diabetes in 2015 and projection for 2040 (IDF, 2015)

	2015	2040
Approximate number of people (million)	415	642
Approximate global percentage of diabetes prevalence (%)	9	10

Projections have shown that this increasing diabetes prevalence is becoming a serious health problem in Africa compared to other global regions. Although from 2011 to 2015 Africa has shown the lowest number of people (between age 20 and 79 years) living with diabetes compared to other global regions (IDF, 2011; 2013; 2015), the percentage increase (140.8%) by the year 2040 has been predicted to be the highest (**Fig 1.1**) (IDF, 2015). In South Africa for example, the number of adults between ages 20 to 79 years with diabetes in 2012 was approximately 6.3% , when high prevalence has been reported among the black, coloured and Indian population (Amod *et al*., 2012; Erasmus *et al*., 2012), and about 50-85% of diabetic individuals remain undiagnosed, particularly in the rural areas (Amod *et al*., 2012).
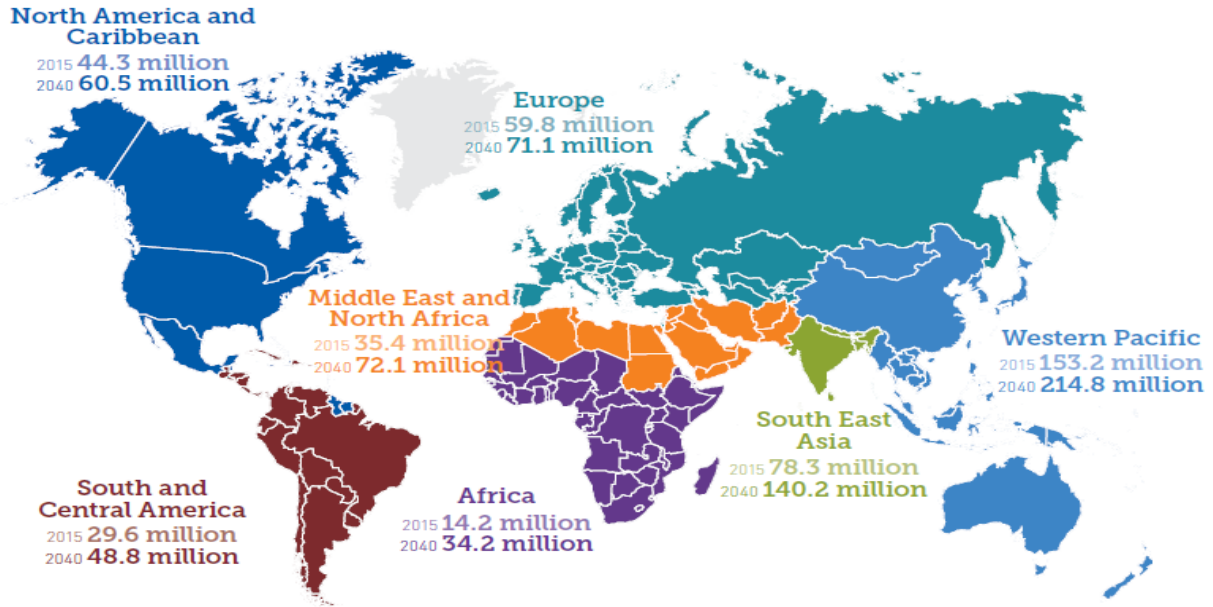
Figure 1.1: Global estimate of people (20-79 years) living with diabetes in 2015 and projections for 2040 (Adapted from IDF Diabetes Atlas, 2015).

As mentioned above, T2D is the most prevalent type of diabetes; accounting for 90% - 95% of all diabetic cases worldwide (Loghmani, 2005; Sheri *et al*., 2010). Additionally, T2D is strongly influenced by dietary related factors, such as unhealthy eating habits, which is a major problem in our present society. Our present study was therefore centered on T2D. Type 2 diabetes (T2D) like other types of diabetes presents persistent hyperglycemia as a major diagnostic manifestation, which is exclusively due to a derangement in glucose metabolism (Aronoff *et al*., 2004). In order to understand the etiology and pathophysiology and management of type 2 diabetes, it is rational to first review and understand the normal physiological metabolism of carbohydrate and glucose.

1.2.4   Carbohydrate metabolism and blood glucose regulation

The major source of dietary glucose for humans is starch, which releases glucose upon complete digestion or hydrolysis. Glucose is majorly absorbed from the small intestine into circulation thus influencing postprandial blood glucose. Therefore, the degree of glucose absorption and the blood level of postprandial glucose can be influencing factors in hyperglycemia-related disorders

like diabetes, obesity and related metabolic diseases (Dhital *et al*., 2013). Glucose is completely and rapidly absorbed from the small intestine, but absorption capacity may be different for different intestinal segments. Previous studies suggested that the highest glucose absorption capacity is exhibited by the proximal (Rider *et al*., 1967) or mid small intestinal segment (Lavin, 1976). Another study conducted on female chicks suggested that the duodenum may account for most glucose absorbed (Riesenfeld *et al*., 1980).

Furthermore, gastric emptying and digesta transit can also affect nutrient (including glucose) absorption from the small intestine (Islam and Indarjit, 2012). Gastric emptying rate means the rate at which nutrients or food is emptied from the stomach to the small intestine, while digesta transit rate is the rate at which digesta in the gut moves through the intestine. It has been previously reported that delayed gastric emptying and accelerated intestinal transit can result in reduced intestinal nutrient absorption (Salminen *et al*., 1984; Shafer *et al*., 1987). Anti-diabetic drug like acarbose have also been suggested to reduce or control postprandial blood glucose by partly delaying gastric emptying (Ranganath *et al*., 1998).

After absorption, glucose immediately circulates in the blood thus causing elevated blood glucose. Accordingly, the human normal physiology is equipped with several interrelated mechanisms to adequately normalize blood glucose level and regulate blood glucose homeostasis (**Fig. 1.2**). Ideally, blood glucose level or concentration is mainly determined by the amount of glucose entering into the blood and the counter-balancing of glucose removal from circulation. Three major metabolic processescontribute to blood glucose pool; dietary intestinal glucose absorption during fed state, glycogenolysis (hepatic glycogen release of glucose) and gluconeogenesis (glucose production primarily from lactate and amino acid precursors) during fasting state (Aronoff *et al*., 2004). On the other hand when blood glucose rises due to metabolic processes that contribute to systemic glucose pool, it is counter-balanced by two major metabolic processes that facilitates the clearance of circulating glucose: glycogenesis (hepatic glycogen synthesis from glucose for storage) and glucose uptake by peripheral tissues for metabolic functions (Aronoff *et al*., 2004). Glucagon hormone is the major hormone that up regulates metabolic processes like glycogenolysis and gluconeogenesis, which favours systemic glucose appearance. Insulin, on the other hand, is the major hormone that up regulates metabolic processes like glycogenesis and peripheral glucose uptake, which favours systemic glucose

disappearance (Aronoff *et al*., 2004). This bi-hormonal glucose homeostasis model functions to maintain a normal range of circulating glucose concentration.

(a)   (b)
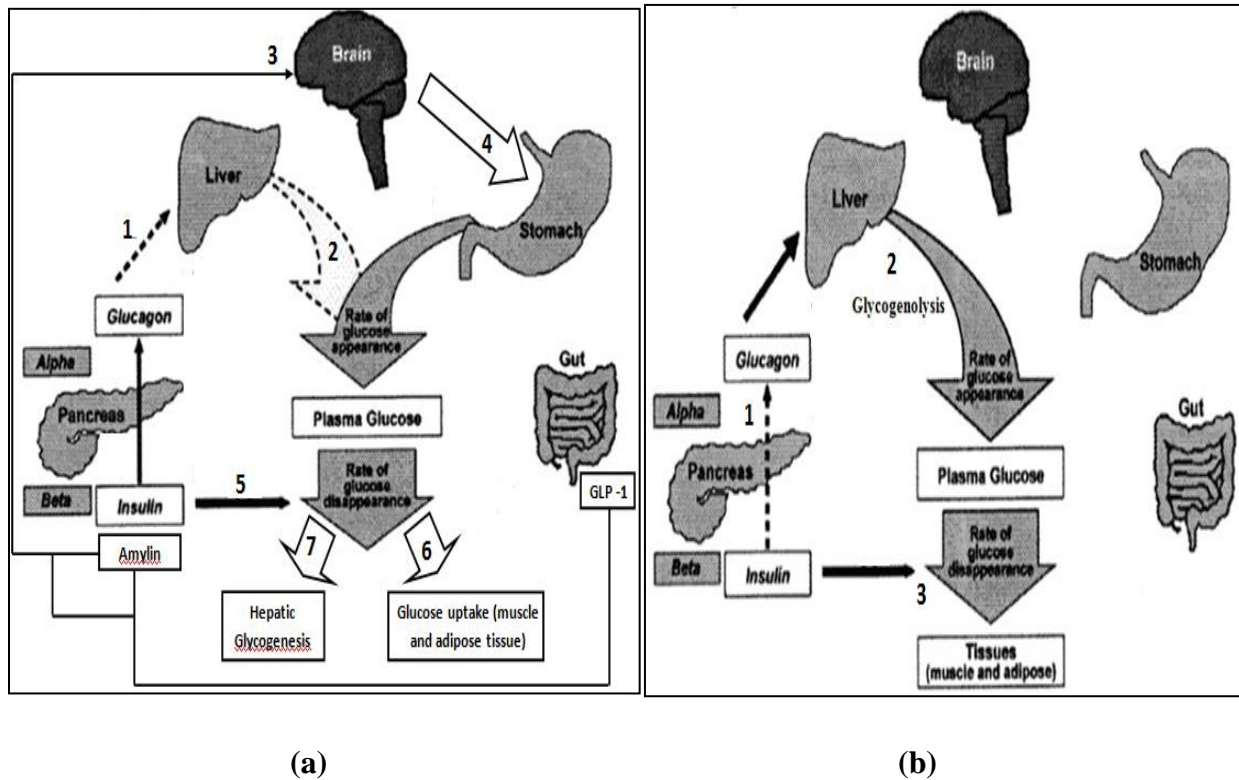
Figure 1.2: Normal regulation of glucose homeostasis. In a fed state (a): Insulin, amylin and GLP- suppresses glucagon secretion and action (1), which inhibits hepatic glucose production (2). Amylin and GLP-1 via a neural pathway (3) reduces gastric emptying rate (4). Insulin promotes circulating glucose disappearance (5) via glucose uptake in peripheral tissue (6) and hepatic glycogenesis (7). In the fasting state (b): Insulin suppression on glucagon is down regulated (1) and glucagon stimulates hepatic glycogen break down (glycogenolysis) (2). Insulin stimulates glucose uptake into peripheral tissues (muscle and adipose) (3).

During fasting state (**Fig. 1.2**), circulating glucose is cleared at a constant rate. In order to counter-balance circulating glucose disappearance, the body is required to produce glucose endogenously. In the first 8 to 12 hours of fasting, glucagon released by the pancreatic alpha

cells stimulates hepatic glycogenolysis, which is the major or sole source of endogenous glucose. But after prolonged fasting, endogenous glucose is additionally made available by hepatic gluconeogenesis, which is also upregulated by glucagon hormone (Aronoff *et al*., 2004). After feeding (**Fig. 1.2**), blood glucose reaches its post-meal peak value, but gradually decreases with time and eventually returns to fasting level, due to circulating glucose clearance. Insulin is the major glucoregulatory hormone that is secreted by the pancreatic beta cells during fed state and when blood glucose elevates. Insulin hormone enhances circulating glucose removal by stimulating hepatic glycogenesis and storage of glycogen as well as glucose uptake in peripheral tissue (majorly skeletal muscle and adipose tissues), while suppressing the secretion and action of glucagon hormone (Gerich *et al*., 1974; Wallum *et al*., 1992; Gerich, 1993). Glucose entering peripheral tissues especially muscle and fat tissue is majorly metabolized at cellular level via an initial glycolytic process to provide chemical energy required for cellular function or used as a precursor for fatty acid synthesis (Aronoff *et al*., 2004).

The insulin-mediated glucose uptake in peripheral tissues, especially muscle tissue is a complex signalling pathway that is made up of a cascade of enzymes and signalling factors at cellular or molecular level (**Fig. 1.3**). Glucose is the major endogenous stimulus of insulin secretion, which enters into the blood after secretion. Once insulin gets into circulation, it acts as a ligand that binds to the extracellular α-subunit of membrane-bound insulin receptor molecule (glycoprotein) to form an insulin receptor-ligand complex (Van Obberghen *et al*., 2001). Binding of insulin causes a conformational change on the intracellular catalytic domain (β-subunit) of insulin receptor molecule, which results in the activation of tyrosine kinase unit of the catalytic domain (Van Obberghen *et al*., 2001; Chang *et al*., 2004). Activated tyrosine kinase then phosphorylates the tyrosine unit of intracellular substrates such as the insulin receptor substrate family (IRS 1, 2, 3 and 4). Phosphorylated IRS family then display binding sites for numerous signalling partners, such as Phosphoinositide 3-kinase (PI3-K) and Mitogen-activated Protein Kinase (MAP-Kinase), which both play major roles in the metabolic function (glycogenesis, peripheral tissue glucose uptake and glucose oxidative metabolism) and mitogenic function (cell growth and gene expression regulation) of insulin respectively (Chang *et al*., 2004; González-Sánchez & Serrano-Ríos, 2007).
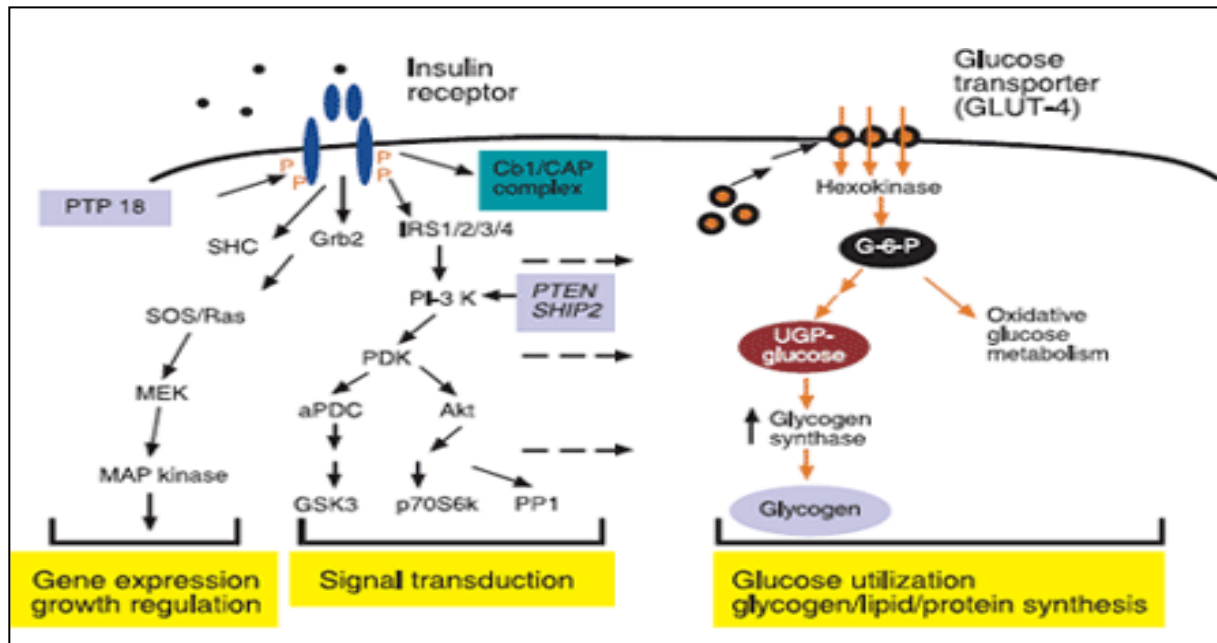
Figure 1.3: Cellular insulin signalling pathway and insulin-mediated glucose uptake (Adapted without permission from González-Sánchez & Serrano-Ríos, 2007).

Phosphoinositide 3-kinase (PI3-K) is activated on binding to IRS-1 and initiates a downstream cascade of events leading to activation of Akt (a serine/threonine protein kinase identified as one of the downstream targets of activated PI3-K) (González-Sánchez & Serrano-Ríos, 2007). Activated Akt induces the translocation of glucose transporter type 4 (Glut-4) to the plasma membrane and influx of glucose for glycogen synthesis, glycolysis, fatty acid synthesis etc (Lizcano & Alessi 2002; Chang *et al*., 2004; González-Sánchez & Serrano-Ríos, 2007).

1.2.5 Etiology and pathophysiology of T2D

In the premature stage and during the early progression of T2D (pre-diabetes), the action of insulin becomes abnormal, which is observed as the loss of timeous postprandial response of insulin (Kahn, 2000). At this stage, an individual may show blood glucose level that is higher than normal but not high enough to be classified as full-blown diabetes. Progressively, insulin resistance in peripheral tissues, coupled with partial pancreatic beta cell dysfunction as well as

reduced availability and potency of insulin contribute to the clinical features and manifestation of persistent or chronic hyperglycemia in T2D (Toft-Nielsen *et al.*, 2001; DeFronzo, 2004).

Insulin resistance can be described as a pathological condition in which cells do not adequately respond to the normal signalling and action of insulin hormone, which predisposes an individual to the development of T2D. Insulin resistance can manifest as failure of insulin to facilitate circulating glucose uptake in peripheral tissues for energy production via glycolysis and TCA cycle or glycogenesis. It can also manifest as failure to suppress the secretion and action of glucagon as well as metabolic processes (glycogenolysis and gluconeogenesis) that promotes circulating glucose appearance andhyperglycaemia (Olefsky & Nolan, 1995). Additionally, since insulin is also functional in the regulation of physiological fat metabolism, insulin resistance can also cause a defect in fat metabolism, which can be linked to the development of several diabetes-related metabolic disorders or complications such as obesity, hypertension, hyperlipidemia, atherosclerosis and metabolic syndrome (Kahn and Flier, 2000; Haag & Dippenaar, 2005; Rabol *et al.*, 2011).

Insulin resistance can occur at any stage of insulin signalling pathway starting from the binding of insulin to insulin receptor to the insulin-mediated Glut-4 translocation and cellular influx of glucose molecules (Olefsky & Nolan, 1995; Petersen & Shulman, 2006). A disorder in the insulin signalling will result inreduced insulin-induced glucose uptake in peripheral tissues and eventually, hyperglycemia. It has been previously reported that patients with non-insulin-dependent diabetes mellitus possessed insulin receptors with diminished autophosphorylation (kinase) activity of the catalytic domain, which contributed to the severity of fasting hyperglycemia (Olefsky & Nolan, 1995). Additionally, the inhibition of insulin-stimulated IRS–1 tyrosine phosphorylation and the subsequent suppression of IRS-1–mediated activation of PI3-K can also result in reduced sensitivity of peripheral tissues to insulin action (Petersen and Shulman, 2006).

As a result of insulin resistance in peripheral tissue, the pancreatic beta cells adopt a compensatory role (secretion and release of more insulin into the blood) to accommodate for the non-responsiveness of cells to insulin action. This will eventually lead to excessive levels of circulating insulin relative to glucose level in the blood, a condition known as hyperinsulinemia (**Fig. 1.4**).
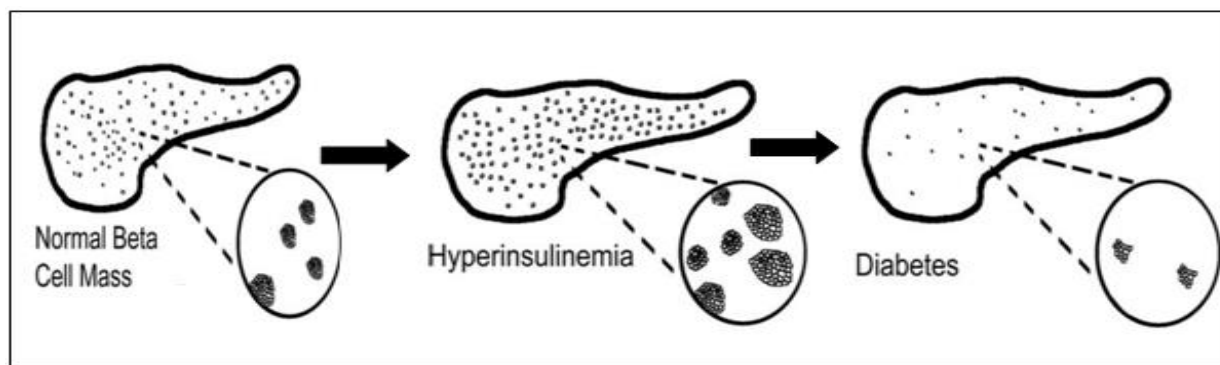
Figure 1.4: Schematic diagram showing the changes in beta-cell mass and insulin secretion accompanying progression of T2D (Adapted without permission from Souza *et al*., 2006).

Hyperinsulinemia promotes elevated energy storage in adipocytes and formation of fatty tissues, which may increase risk of hypertension, obesity, dyslipidemia hypertriglyceridemia, and glucose intolerance, collectively referred to as metabolic syndrome (Modan *et al*., 1985; Shanik *et al*., 2008). Additionally, the compensatory role adopted by the pancreatic beta-cell due to insensitivity of cells to insulin action may gradually become exhaustive. Eventually, this adversely affects the pancreatic beta-cell function and physiology; a condition that is referred to as partial pancreatic beta-cell dysfunction (**Fig. 1.4**) (Cnop *et al*., 2005; Souza *et al*., 2006).

In a previous study, Ogawa *et al*. (1992) demonstrated that insulin resistance, beta-cell dysfunction and impaired insulin secretion were inter-related in the development of T2D (Ogawa *et al*., 1992). Their study showed that despite the presence of insulin resistance in both diabetic and non-diabetic rats, the mass of the islet and insulin secretion of diabetic rats was lesser compared to non-diabetic rats, which suggested the presence of impaired insulin secretion in the diabetic animals due to beta cell derangement. Accordingly, they concluded the co-existence of insulin resistance and pancreatic beta-cell in the pathophysiology of T2D.

Additionally, abnormal gastric emptying has been observed during type 1 or type 2 diabetes (Aronnof *et al*., 2004).

1.2.6 Role of gastric emptying in the pathophysiology of T2D

Gastric emptying is normally delayed in the fed state by the action of amylin and GLP-1 in order to regulate postprandial glycemia (**Fig. 1.1**). Although the prevalence is controversial, studies have suggested that gastric emptying is often accelerated, especially during the early stages of T2D (Phillips *et al*., 1991; Horowitz *et al*., 1996). In our recently published study, we observed that induction of T2D in rats significantly increased gastric emptying rate of normal rats (Chukwuma & Islam, 2015). Accordingly, we hypothesized that accelerated gastric emptying may partly contribute to the frequent hunger (polyphagia) that is observed during diabetes, because of the previously reported correlation between faster gastric emptying and reduced satiety or increased food intake in healthy male subjects (Zhu *et al*., 2013). Additionally, since the concentration of postprandial glucose and insulin secretion is related to gastric emptying rate (Horowitz *et al*., 1993), accelerated gastric emptying may translate into a faster increase of postprandial blood glucose concentration. This is mostly accompanied by apoorly timed insulin delivery, which may eventually aggravate or worsen postprandial hyperglycemia (Phillips *et al*., 1991).

1.2.7 Complications of T2D

Due to uncontrolled hyperglycaemia, more than 50% people suffer from one or more diabetic complications including diabetic nephropathy, diabetic retinopathy, diabetic cardiomyopathy, diabetic neuropathy and diabetic food disease (Litwak *et al*., 2013). Most diabetic complications are responsible for the increasing morbidity and mortality associated with diabetes. Diabetic complications are broadly classified into acute and chronic complications.

i)   Acute complications

The acute complications include diabetic ketoacidosis, nonketotic hyperglycemic coma as well as hypoglycaemia and hypoglycaemic reactions, which are basically due to metabolic and glucose level alterations (Henry, 1987). Diabetic ketoacidosis is due to insulin deficiency in the presence of hyperglycaemia, which leads to increase in lipolysis and ketone bodies production,

and eventually increase in blood acidity (blood pH ≤ 7.3) (Faich *et al*., 1983). Diabetic ketoacidosis can also lead to diabetic coma (Faich *et al*., 1983, Henry, 1987).

Non-ketotic hyperglycemic coma is also called hyperosmolar hyperglycemic non-ketotic coma. It is also due to insulin deficiency in the presence of hyperglycemia, although the insulin deficiency is not enough to cause increased lipolysis and ketone bodies production. However, the presence of hyperglycemia causes elevated serum osmolality, which leads to polyuria, excessive dehydration, seizures and coma (Loewen & Haas, 1991). Most of the long-term or chronic diabetic complications are more detrimental than acute diabetic complications (Fowler, 2008).

ii)   Chronic complications

Chronic diabetes complications are associated with direct and/or indirect severe hyperglycemic effect on the human vascular tree, which are the major causes of morbidity and mortality in diabetes. They can be sub-classified into the microvascular and macrovascular complications.

(a)   Microvascular complications

Microvascular diabetic complications are associated with small vessels such as capillaries-related problems. Several theories have been suggested in an attempt to elucidate the pathophysiology of microvascular complications associated with T2D. The polyol pathway and oxidative stress are some of them (Fowler, 2008).

The intracellular polyol pathway consists of an initial aldose reductase-catalyzed reaction that may be involved in the development of type 2 diabetic complications. Aldose reductase catalyzes the conversion of glucose to sorbitol. In hyperglycemic state, more sugar molecules flux through the polyol pathway, which causes accumulation of sorbitol in cells. Sorbitol accumulation in cells can result in osmotic stress, which has been hypothesized as an underlying mechanism in the pathophysiology of most diabetes-associated microvascular complications (Gabbay, 1975).

Furthermore, hyperglycemia-induced oxidative stress has been implicated in the symptoms, progression and complications of T2D (Giugliano *et al*., 1996; Pourghassem-Gargari *et al*., 2011). Persistent hyperglycemia is responsible for elevated superoxide ion production, glucose oxidation, protein glycosylation, formation of advanced glycation end products (AGEs) and lipid peroxidation, which facilitate the production of pro-oxidants such as free radicals and reactive oxygen species (Wiernsperger, 2003). Oxidative stress sets in when the generated pro-oxidants surpasses the body's antioxidant defence system. Reactive oxygen species play a major role in cellular damage during oxidative stress, which contributes to diabetic microvascular complications. Animal studies have demonstrated that treatment with vitamin E, a known anti-oxidant molecule may be useful in ameliorating diabetes-associated microvascular dysfunction (Kunisaki *et al*., 1995).

The notable forms of diabetes-associated microvascular complications are called diabetic retinopathy, diabetic nephropathy and diabetic neuropathy.

- **Diabetic retinopathy**

Diabetic retinopathy may be the major cause of blindness in developing and developed countries (Fowler, 2008; Singh *et al*., 2008). It is characterized by the dysfunction of retina and/or the macula, which can lead to visual problem and eventually, partial or total blindness in type 2 diabetic patients. The loss of pericytes and microaneurysm formation are the predominant marker for diabetic retinopathy (Fowler, 2008; Singh *et al*., 2008).

The pathogenesis of diabetic retinopathy is still not clear and remains a subject of debate. However, there are several theories which explain the pathophysiology of diabetic retinopathy. Sorbitol-related osmotic stress and electrolyte imbalance due to increased metabolic flux of the polyol pathway are important catalysts of retinal pericytes death and microaneurysm formation (Kubawara and Cogan, 1962; Fowler, 2008). Also, the normal hemodynamics of the retina can be altered due to distortion of the inner blood retinal barrier and extracellular fluid deposition (Williamson & Kilo, 1984; Ferris & Patz, 1984). Furthermore, AGEs and glycated proteins can also cause injury to pericytes leading to loss of pericytes. There are evidences in animal studies where AGEs was associated with the loss of pericytes and formation of microaneurysms (Fong *et al*., 2004). Additionally, growth factors

such as vascular endothelial growth factor (VEGF), growth hormone, and transforming growth factor β have also been suggested to participate significantly in the development of diabetic retinopathy. Notable increases in VEGF production have been reported in diabetic retinopathy (Aiello *et al*., 1995; Keenan *et al*., 2007), perhaps due to hypoxia. Additionally, previous studies have also reported that the progression of retinopathy was impaired in animal models due to suppression of VEGF production (Aiello *et al*., 1995).

- **Diabetic nephropathy**

Diabetic nephropathy is the leading cause of renal failure in majority of elderly people seeking kidney disease therapy (Parchwani & Upadhyah, 2012). It is characterized by persistent occurrence of increased urinary albumin excretion (microalbuminuria and macroalbuminuria) in diabetic patients, without manifestation of other renal disease symptoms (Gross *et al*., 2005). Early clinical diagnostic markers of diabetic nephropathy include microalbuminuria (30-300 mg/day), glomerular hyperfiltration, glomerular and renal hypertrophy, increased glomerular basement membrane thickness, microaneurysm formation and mesangial nodule formation (Kimmelsteil-Wilson bodies) (Gross *et al*., 2005, Fowler, 2008). Without therapeutic intervention microalbuminuria in diabetic patients progresses into an advanced stage of overt diabetic nephropathy, characterized by proteinuria, macroalbuminuria (>300 mg/day), progressive deterioration in the rate of glomerular filtration and creatinine clearance as well as glomerulosclerosis (Gross *et al*., 2005, Fowler, 2008; Parchwani & Upadhyah, 2012).

Genetic factor, race and gender susceptibility, high-protein diet, smoking, aging, hypertension, hyperglycemia and hyperfiltration have been identified as notable risk factors in the development and progression of diabetic nephropathy (Ayodele *et al*., 2004). However, theories put forward suggest that growth factor and cytokines; hemodynamic factors such as Angiotensin II and Endothelin; protein glycosylation; AGEs formation; polyol and protein kinase C pathways are involved in the underlying mechanisms behind the pathophysiology of diabetic nephropathy (Parchwani and Upadhyah, 2012).

- **Diabetic neuropathy**

According to the American Diabetes Association (ADA), diabetic neuropathy is described as the occurrence of clinical symptoms of peripheral nerve dysfunction in diabetic individuals without the presence of other nondiabetes-related peripheral nerve dysfunction (ADA, 2007). Like other diabetes-associated microvascular complications, the risk of developing diabetic neuropathy is a function of the intensity and persistence of hyperglycemia, although genetic susceptibility is also a risk factor.

al Although the actual nature of injury to the peripheral nerves by hyperglycemia and the underlying pathogenic mechanisms still remains unclear, several theoretical mechanisms have been put forward to explain the pathogenesis and pathophysiology of T2D-associated neuropathy (Head, 2006; Fowler, 2008). These include accumulation of polyol due to increase of metabolic flux of the polyol pathway; oxidative stress and AGEs-mediated peripheral nerve injury; increased activity of protein kinase C pathways; and reduced levels of neurotrophic agents (Boulton *et al*., 2005; Ziegler, 2008; Fowler, 2008). Peripheral neuropathy in diabetes may present in different forms, including sensory, focal/multifocal, and autonomic neuropathies. These may lead to morbidities such as amputations due to foot ulceration or injury, loss of ankle reflex, gastroparesis, anhidrosis, bladder dysfunction, erectile dysfunction, myocardial ischemia and eventually death (Abbott *et* ., 2002; Maser *et al*., 2003; Boulton *et al*., 2005).

(b)   Macrovascular complications

These are complications associated with large vessels like arteries and veins, which are mostly, linked to cardiovascular events in type 2 diabetic individuals. Cardiovascular disorders (CVDs) are the leading cause of mortalities in patients with T2D. Data from previous investigations showed that the risk of developing CVDs, excluding other traditional CVDs risk factors like aging, smoking, dyslipidemia, hypertension and obesity is four times higher in type 2 diabetic individuals compared to non-diabetic individuals (Buyken *et al*., 2007).

The principal pathophysiological mechanisms of diabetic macrovascular complications, especially CVDs have been postulated to grossly involve the pathogenic processes of atherosclerosis (Fowler, 2008). Atherosclerosis is characterized by a severe inflammation and injury to the arterial wall in the peripheral or coronary vascular system. This is due to the deposition of fatty material on the arterial inner walls, which causes the lumen of arteries all over the body to narrow down (Insull, 2009). Fatty particles or deposits in the arterial endothelial walls may undergo Angiotensin-induced oxidation, while monocytes infiltrate arterial wall to form macrophages after differentiating. Macrophages formation causes T-lymphocyte-associated proliferation of arterial wall smooth muscles, which eventually lead to an acute vascular infarction (Maser *et al.*, 2003).

Furthermore, hyperglycemia-induced free radical formation in arterial platelets can impair the generation of nitric oxide (an important vasodilator), which promotes atheroma formation and arterial platelet aggregation and adhesion. This condition, in the presence of impaired fibrinolysis, can further aggravate the risk of cardiovascular events in T2D individuals (Fowler, 2008).

1.2.8   Management of T2D

Dietary and lifestyle modifications are commonly recommended for people with T2D, especially in less severe cases and for long term management programs. However, pharmacological agents are usually introduced when dietary and lifestyle modifications are not effective in controlling hyperglycemia, especially in chronic or later stage of T2D.

i)   Use of therapeutic drugs

The major rationale behind the use of pharmacological agents in the management of T2D sterns from the severity of hyperglycemia in patients. Pharmacological agents or therapeutic drugs for the management of T2D act promptly to control re-occurring incidences of abnormal high blood glucose in chronic T2D conditions. There are several pharmacological agents currently available for the management of T2D, which are sub-divided into different classes depending on the

mechanism of action employed to ameliorate hyperglycemia (Tsang, 2012). In this section, the mode of action and side effects of some anti-diabetic drugs/therapies like biguanides, sulfonylureas, thiazolidinedione, alpha glucosidase inhibitors, dipeptidyl-peptidase (DPP) IV inhibitors and incretin-based therapies are discussed (**Fig. 1.5**).

(a)   Biguanides

Although the mechanism of action is not completely known, it has been reported that biguanides lower blood glucose by suppressing hepatic glucose production (especially gluconeogenic processes), while enhancing circulating glucose uptake in peripheral tissue and decreasing intestinal glucose absorption (Collier *et al*., 2006). Metformin is the most recent and most frequently used biguanide in the market, commonly used by overweight and obese patients with T2D. Previous studies have suggested that metformin suppresses gluconeogenesis via the activation of AMP-activated protein kinase, which is a key regulatory enzyme involved in the expression of genes that regulate gluconeogenesis (Kim *et al*., 2008). Additionally, metformin has also been reported to increase fatty acid oxidation and decreases plasma lipid levels (DeFronzo *et al*., 1991; Collier *et al*., 2006), which can enhance weight loss in obese and lean individuals with non-insulin-dependent diabetes mellitus (DeFronzo *et al*., 1991). However, metformin medication in some individuals is associated with some gastrointestinal side effects such as nausea, abdominal spasm, diarrhea and bloating (Bailey and Turner, 1996). Metformin medication can also cause lactic acidosis, especially in elderly diabetic individual suffering from renal impairment; hence these individuals are advised to use metformin with caution (Misbin *et al*., 1998; Collier *et al*., 2006).

(b)   Sulfonylureas

Current drugs belonging to this class of anti-diabetic agents include gliclazide, glibenclamide, glipizide and glimepiride, with glibenclamide being the most popular. They lower blood glucose by stimulating endogenous insulin secretion in the pancreas and improving beta cell functions (Koski, 2004; Chiniwala & Jabbour, 2011; Tsang, 2012). Sulfonylureas bind strongly to beta

cells via membrane-specific receptors, which signal the release of insulin from secretory granules within the beta cells (Tsang, 2012). However, direct induction of insulin secretion by beta cells regardless of blood glucose levels can increase the risk of hypoglycemia, which is a the major adverse effect of sulfonylureas medication (van Staa *et al*., 1997; Chiniwala and Jabbour, 2011). Elderly patients have an increased risk of 38% in developing sulfonylureas-associated hypoglycemia compared to younger patients (van Staa *et al*., 1997).
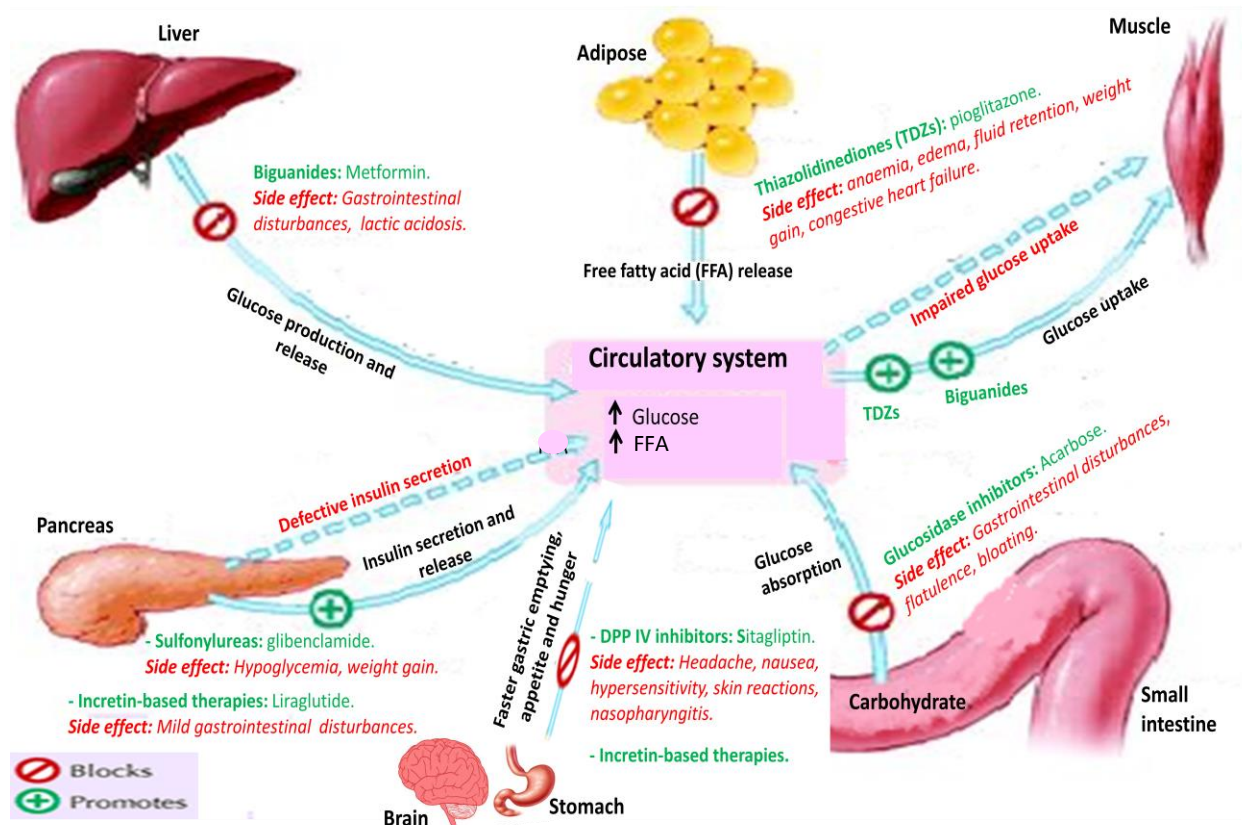
Figure 1.5: Diagram showing some current anti-diabetic drugs, their mechanisms of action and side effects. (Adapted and modified from Cheng & Fantus, 2005)

(c)   Thiazolidinedione

Thiazolidinediones are earliest anti-diabetic drugs that were produced to tackle the problem of insulin resistance in T2D (Yki-Järvinen, 2004). They are referred to as insulin sensitizers,

because they enhance insulin sensitivity in peripheral tissues. Thiazolidinediones improve insulin sensitivity via a basal route that indirectly involvesthe regulation of lipid metabolism by peroxisomes proliferator-activated receptor gamma (PPAR-γ) (Yki-Järvinen, 2004; Hammarstedt *et al.*, 2005). Peroxisomes proliferator-activated receptor gamma (PPAR-γ) is a transcription factor that is involved in the insulin-dependent regulation of carbohydrate and lipid metabolism. Thiazolidinediones act as a ligand that strongly binds to (PPAR-γ), thus activating it. Activated PPAR-γ upregulates insulin-mediated glucose uptake in the muscle and fat tissues; expression of adipocyte insulin-sensitizing hormone; and adipocyte differentiation, while suppressing lypolytic processes (Yki-Järvinen, 2004; Hammarstedt *et al.*, 2005).

The major classes of thiazolidinediones are pioglitazone and rosiglitazone. However, rosiglitazone has been recommended for restriction by the Food and Drug Administration (FDA) due to some reported associated cardiovascular events (Yoon *et al.*, 2006). On the other hand, pioglitazone is not associated with hypoglycemic effects and can be well tolerated by elderly patients. However, several side effects such as anaemia, edema, fluid retention, weight gain and congestive heart failure have been reported to be associated with pioglitazone medication. Thus, pioglitazone should be avoided by type 2 diabetic patients and elderly people with congestive heart failure, hepatic impairments and patients with class III-IV heart failure (Inzucchi, 2002).

(d) **Alpha glucosidase inhibitors**

Alpha glucosidase inhibitors inhibit the action of carbohydrates digestive enzymes such as alpha glucosidase and alpha amylase on carbohydrate breakdown, thereby limiting postprandial glucose absorption in the small intestine (Patel *et al.,* 2012). This class of anti-diabetic pharmacological agents is therefore most effective in controlling postprandial hyperglycemia. Studies suggest that alpha glucosidases inhibitors are carbohydrate analogues, which inhibit the action of carbohydrate digestive enzymes in a competitive fashion (Horii *et al.,* 1986; kim *et al.,* 1999).

Acarbose, voglibose and miglitol are the examples of some the commonly used alpha glucosidase inhibitors, although acarbose is the most popular one. Besides suppressing carbohydrate breakdown, studies have shown that acarbose may also exert glycemic control by

delaying gastric emptying, which may reduce small intestinal glucose absorption (Ranganath *et al*., 1998). However, the use of alpha glucosidase inhibitorsis limited, due to frequent incidences of gastrointestinal side effects such as diarrhea, bloating and flatulence (Cheng & Fantus, 2005).

(e)   Dipeptidyl-peptidase (DPP) IV inhibitors

This class of anti-diabetic drugs inhibits dipeptidyl peptidase (DPP) IV, which is an antagonist of incretin hormones like glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (Pratley and Salsali, 2007). Incretin hormones promote beta cell mass, suppress glucagon secretion, delay gastric emptying and induce satiety (Pratley and Salsali, 2007). Accordingly, the action of DPP IV inhibitors preserves the activity of incretin hormones, thereby enhancing postprandial glycemic control. Sitagliptin is the first DPP IV inhibitor that was introduced into the market in 2006, followed by vildagliptin, saxagliptin, linagliptin and alogliptin. These drugs are well tolerated and have low risk of causing hypoglycemia. However, side effects like headache, nausea, hypersensitivity, skin reactions and nasopharyngitis have been reported with DPP IV inhibitor medication (Gallwitz, 2013). Additionally they are relatively expensive compared to other commonly used oral hypoglycemic drugs .e.g metformin.

(f)   Incretin-based therapies

These are analogues or agonists of incretin hormone, which mimic the action GLP-1, thereby by enhancing incretin-based glycemic control (Stonehouse *et al*., 2012). Examples include exenatide and liraglutide. Incretin-based therapies present no severe risk of hypoglycemia, but may present mild gastrointestinal disturbances (Garber, 2011). Incretin-based therapies may also be useful in cardiovascular and hepatic health (Stonehouse *et al*., 2011). However, very little is known about incretin-based therapy so far.

ii) Exercise and physical activities

Exercise and physical activities play a significant role in the management of T2D because they improve carbohydrate and lipid metabolism, assist in glycemic and weight gain control and ameliorate lipid-induced insulin resistance associated with obesity, T2D and metabolic syndrome (Winnick *et al*., 2008; Colberg *et al*., 2010). Previous studies have presented consistent evidence on the therapeutic effect of both acute and chronic exercise on T2D individuals. For instance, aerobic exercise, which is the most popular type of physical exercise, has been reported to improve insulin sensitivity in patients starting from the first week of exercise (Winnick *et al*., 2008). Other studies have demonstrated that physical exercise reduces total and LDL-cholesterol but increases HDL-cholesterol (Kadoglou *et al*., 2007); and is also useful in the prevention and management of weight gain and T2D-related complications such as hypertension and cardiovascular events (Sigal *et al*., 2006).

iii) Dietary management

Detrimental positive energy balance and weight gain resulting from unhealthy dietary habits is a major contributor to overweight or obesity, which can increase the risk of developing serious health problems, including T2D, cardiovascular diseases, hypertension and metabolic syndrome (Artham *et al*., 2009). Accordingly dietary adjustment is one of the first therapeutic recommendations in the managements of T2D. According to the Society for Endocrinology, Metabolism and Diabetes of South Africa (SEMDSA) guidelines for the management of T2D, type 2 diabetic patients should consume less saturated fat and also reduce the intake of high-calorie sugars like sucrose to about 10% of total energy intake per day (Amod *et al*., 2012). This is because despite the fact that sugar consumption will aggravate hyperglycemia in patients, chronic consumption has been consistently shown to be strongly associated with detrimental health outcomes such as elevated food intake, body weight gain and visceral adiposity (Astrup *et al*., 2002; Hu & Malik, 2010; Stanhope *et al*., 2011;); reduced insulin sensitivity and lipid dysregulation (Thorburn *et al*., 1989; Elliott *et al*., 2002); and the risk of developing T2D, obesity, metabolic syndrome and cardiovascular diseases (Schulze *et al*., 2004; Montonen *et al*., 2007; Bantle, 2009; Stanhope, 2012).

Major dietary adjustments in diabetics that has recently gained global acceptance is the use of sugar substitutes such as non-nutritive and nutritive (reduced-calorie) sweeteners to replace sugar in diabetic diets (Talbot & Fisher, 1978; Brown *et al*., 2010), because they are known to have little or no glycemic or caloric value compared to sucrose; thus, are useful for glycemic control in diabetics.

1.2.9 Non-nutritive and nutritive sweeteners

Sweetener is the common name for sugar replacers or sugar substitute. They are basically used in place or together with table sugar (sucrose). Their sweetness range from slightly less sweet to several thousand times sweeter than sucrose and are broadly classified as either non-nutritive or nutritive (reduced-calorie) sweetener (Fitch & Keim, 2012).

i)   Non-nutritive sweeteners (NNSs)

As the name imply, these sweeteners do not provide the body with any sort of nutrition. In fact they cause a zero caloric effect upon consumption. Their sweetening power range from about 30 – 13000 times higher than sucrose, and as such, are also referred to as intense sweeteners (O'Donnell & Kearsley, 2012; Fitch & Keim, 2012). They are used in relatively smaller amounts compared to reduced-calorie sweeteners, due to their intense sweetening ability. At high amounts, they exhibit a sweetness profile that is characterized by an unpleasant after taste. Non-nutritive sweeteners are sometimes referred to as artificial sweeteners because of their synthetic origin, except for some high potency natural sweeteners like stevia, thaumatin, mogrosides, brazzein and monatin, which are derived from plants (Lindley, 2012). The commonly used NNSs include saccharin, aspartame, sucralose, acesulfame potassium (Ace-K), cyclamate, neotame and stevia. Although artificial sweeteners are widely used in several products and as table top sweeteners, with endorsement from different food regulatory bodies, it is often difficult to obtain or verify a universal regulatory status regarding the use of these sweeteners. This is due to the continuously changing food regulatory processes, which is subject to different interpretations by various regulatory bodies in different countries.

(a)   **Saccharin**

Saccharin is the oldest NNSs, which was accidentally discoveredin 1897 by Constantine Fahlberg, while working in the laboratory of Ira Remsem, Hopkins University, United States (DuBois, 2012). It is a heterocyclic white crystalline compound (**Fig. 1.6**), which is about 300 times sweeter than sucrose (Fitch &Keim, 2012). It is highly absorbed but not metabolized in the body; hence has a zero caloric effect (DuBois, 2012). Absorbed saccharin is excreted in the urine. Saccharin has been approved by the United States FDA for general use except in meat and poultry, with an ADI of 15 mg/kg bw (Fitch & Keim, 2012).
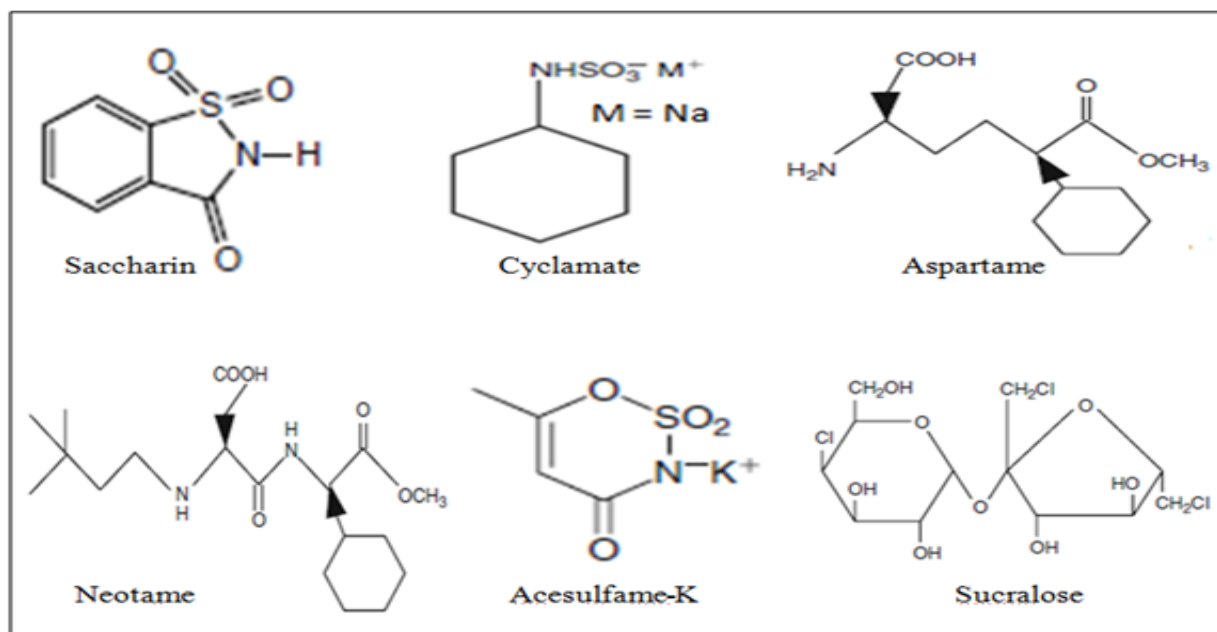
Figure 1.6: Chemical structures of some non-nutritive sweeteners (structures adapted without permission from O'Donnell & Kearsley, 2012).

(b)   **Cyclamate**

Cyclamate is a white crystalline sodium or calcium salt of cyclohexylsulfamic acid (cyclamic acid) (**Fig. 1.6**). The sweetening property of Cyclamate was first reportedin 1944 by Sveda and Audrieth at the University of Illinois (DuBois, 2012). Sodium cyclamte is about 30 to 50 times

sweeter than sucrose (Fitch & Keim, 2012; DuBois, 2012). As a result of its low sweetening power and bitter-salty off taste, sodium cyclamate is often used with other non-nutritive sweetenersin blended combination. Cyclamate is not metabolized in human tissue but can be converted to cyclohexylamine, cyclohexanol and cyclohexane-1,2-diol by the action of large intestinal microflora of some individuals (Bopp & Price, 2001). Absorbed cyclamate and cyclamate metabolite are completely excreted in the urine, post consumption. Cyclamate has gained approval for use in beverages and drink and as a table top sweetener in more than 50 countries but not in the United States (Fitch & Keim, 2012). The ADI for cyclamate approved by JECFA is11.0 mg/kg bw (DuBois, 2012).

(c)   Aspartame

Aspartame is a white crystalline ester of two amino acids, l-phenylalanine and l-aspartic acid, joined by a methyl-ester link (**Fig. 1.6**). It was discovered by Schlatter in 1965 in the laboratories of G.D. Searle (O'Donnell, 2012). Aspartame was first introduced to the US market in 1981 as "NutraSweet", which became a house-hold brand name by the late 80's and early 90's. Aspartame is about 160 to 220 time sweeter than sucrose and characterized by a sweet clean taste, which can improve the synergistic effect with other sweeteners or flavours (Fitch & Keim, 2012; O'Donnell, 2012).

As a dipeptide molecule, aspartame is metabolized or hydrolyzed in the body by digestive peptidases into methanol and its constituent amino acids, l-phenylalanine and l-aspartic acid. Amino acids are absorbed in the intestines to become part of protein metabolism in the body, while methanol is excreted (Ranney & Opperman, 1979; Harper, 1984). Although methanol metabolite may be potentially harmful at toxic levels (200–500 mg/kg body weight), the amount of methanol resulting from aspartame metabolism is relatively very small and harmless compared to the toxic levels (Stegink *et al.*, 1981).

Aspartame is used as a table top sweetener and in several products like yoghurts, confectionaries, beverages, but not suitable for baking, because it is not heat stable. Major food regulatory bodies have established an ADI of 40-50 mg/kg bw for aspartame, but have restricted the use of aspartame for people suffering from phenylketonuria (Fitch & Keim, 2012;

O'Donnell, 2012). This is because sufferers of this disease are deficient of the enzyme phenylalanine hydroxylase, and therefore cannot physiologically metabolize phenylalanine into tyrosine. Accumulation of phenylalanine may result in mental retardation (Ranney & Opperman, 1979; Harper, 1984).

(d)   Neotame

Neotame is an aspartame derivative that was born out of a collaborative research between the French research group, Tinti and Nofre of Claude Barnard University France and The Nutra Sweet Company (Nofre & Tinti, 2000). The purpose of the collaborative research was to develop a novel sweetener with better physical and organoleptic properties as well as lesser production cost compared to aspartame. By 1991 the discovery of an n-[n-(3,3-dimethylbutyl)-l-aspartyl]-l-phenylalanine-1-methyl ester (**Fig. 1.6**), which was later named neotame was reported (O'Donnell, 2012). Neotame is about 7000 to 13000 times sweeter than sucrose and it is characterized by a clean sweet taste, similar to sucrose (Fitch & Keim, 2012; O'Donnell, 2012). It is partially absorbed in the intestine and de-esterified in the body, but does not release phenylalnine or toxic amount of methanol; hence, is suitable for people suffering from phenylketonuria (O'Donnell, 2012). Both absorbed and de-esterified neotame are excreted in the urine and feces. Noetame has been approved by the US FDA as a general use sweetener with an ADI of 18 mg/kg bw, but not suitable in meat and poultry (Fitch & Keim, 2012).

(e)   Acesulfame potassium (Ace-K)

Acesulfame potassium (Ace-K) is a colourless monoclinic crystalline potassium salt of Acesulfame (a dihydro-oxathiazinone dioxides) (**Fig. 1.6**), which was accidentally discovered by Clauß and Jensen in 1967 (Clauß&Jensen, 1973). Ace-K is about 200 times sweeter than sucrose and is characterized by a sweetness profile that does not remain longer than the original taste of the food it sweetens (Klug & Lipinski, 2012; Fitch &Keim, 2012). It is partially absorbed but not metabolized in the body, hence both absorbed and non-absorbed Ace-K completely excreted in the urine and feces. The US FDA has approved an ADI of 15 mg/kg bw for Ace-K (Fitch &

Keim, 2012). Ace-K is used as a table top sweetener, as blended combination with other sweeteners and in products such as pharmaceuticals, bakery food, canned food, sweets and chewing gums.

(f)   Sucralose

Sucraloseis a white powder-like sweetener, derived from sucrose by the replacement of some hydroxyl groups with chlorine to produce a halogenated sugar (**Fig. 1.6**) (Molinary and Quinlan, 2012). The research leading to the discovery of sucralose started in the 1970's by Tate & Lyle, PLC (Fitch & Keim, 2012). Sucralose is about 600 times sweeter than sucrose and it suitable for baking due to its heat stability (Molinary and Quinlan, 2012). Although sucralose is a sucrose derivative, it is partly absorbed but not metabolized in the body. Hence both absorbed and unabsorbed sucralose are excreted in the urine or feaces (Roberts *et al*., 2000). Sucralose was approved by the US FDA in 1998 to be used in desserts and beverages with an ADI of 5 mg/kg bw.

(g)   Stevia

Steviais composed of steviol glycosides-rebaudioside A and stevioside, which are sweet organic compounds, extracted from the leaves of the plant *Stevia rebaudiana Bertoni* (Kinghorn & Soejarto, 1991). Stevia is about 250 times sweeter than sucrose, but characterized by a clean taste that can be bitter at higher quantities (Goyal *et al*., 2010). Stevia is rarely metabolized or absorbed in human, because the β-glycosidic linkages in steviol glycosides cannot be hydrolyzed by human digestive enzymes. However, microbial fermentation can occur in the large intestine to release steviol, which can be absorbed and excreted in the urine as steviol glucuronide, a conjugate of glucoronic acid (Wheeler *et al*., 2008). Stevia has been approved by the US FDA as a general use sweetenerand an ADI of 4 mg/kg bw has been determined by JECFA (Fitch & Keim, 2012).

Despite, the endorsement of these NNSs by several food regulatory bodies, the several life threatening side effects that have been previously reported to be associated with some of

them may still remain a major concern for chronic consumers (Whitehouse *et al*., 2008). Additionally, though it may be safer at ADI dose or lower, most of the consumers are unaware of this. Accordingly, it may not be always possible to adhere to the ADIs, since NNSs are being used in the thousands of food products.

ii)   Nutritive or reduced-calorie sweeteners

Considered as an alternative for sugars inobese and diabetics diets, nutritive or reduced-calories weeteners can be described as sweetening agents that can add calorie when ingested, but relatively lesser compared to sucrose, glucose or other sugars (Fitch & Keim, 2012). They are generally referred to as low glycemic index sweeteners, because they do not increase postprandial glucose level as much as sugars (Livesey, 2003). Most reduced-calorie sweeteners possess relatively less or similar sweetness compared to sucrose or glucose, hence they are used to replace the bulk or volume of sugar in foods (Fitch & Keim, 2012). There are several examples of reduced-calorie sweeteners, but for the purpose of this study the discussion will be limited to a class of reduced-calorie sweeteners known as sugar alcohols or polyols.

1.2.10 Sugar alcohols

Sugar alcohol is a class of reduced-calorie sweeteners mostly derived from simple carbohydrate like monosaccharides and disaccharides by catalytic hydrogenation of their carbohydrate precursors. They possess multiple hydroxyl functional groups without aldehyde (-CHO) or ketone (=CO) functional group that are present in their carbohydrate precursors, and also occur naturally in numerous fruits and vegetables or in the body as metabolites of carbohydrate metabolism (O'Donnell & Kearsley, 2012). Sugar alcohols possess lesser or about similar sweetness compared to sucrose. Their sweetness profile is characterized by a pleasant cooling effect that is void of a bitter or unpleasant after taste. Almost all sugar alcohols are sparingly metabolized and absorbed in the intestine, hence they provide lesser calorie and cause lesser blood glucose rise or insulin response compared to equivalent amount of sugars like sucrose or

glucose (O'Donnell & Kearsley, 2012). However, the glycemic responses of sugar alcohols are different, due to their varying digestibility, absorption capacity and metabolism (**Table 1.2**).

Table 1.2: Physiological and organoleptic properties of sucrose and some commonly used sugar alcohols.

Sweeteners	RGR[a]	RIR[a]	Caloric value (kcal/g)[b]	Relative sweetness (%)[c]	Percentage absorption (% of ingested amounts)[d]	Percentage urinary excretion (% of ingested amounts)[d]	Laxative threshold (g/day)[b]
Sucrose	68	45	4	100	ND	90	~125
Erythritol	0	2	0	70	90	<2	~125
Xylitol	12	11	2.4	95	50	<2	~50
Mannitol	0	0	2.4	50	25	25	~20
Isomalt	9	6	2.4	40	10	<2	~50
Lactitol	5	4	2.4	40	2	<2	~20
Maltitol	45	27	2.4	90	40	<2	~96
Sorbitol	9	11	2.4	50	25	<2	~50
Myo-inositol	ND	ND	ND	50**	ND	ND	ND

[a]Relative glucose response (RGR) and relative insulin response (RIR) as percentage of glucose and insulin responses of oral glucose equivalence (values adapted from Livesey, 2012). [b]Values adapted from Flambeau *et al*., 2012. [c]Values adapted from Sadler and Stowell, 2012, except for myo-inositol (**). [d]Values adapted from Livesey, 2003. ND: Not determined.

Absorbed portion of most sugar alcohols are metabolized to become part of carbohydrate metabolism, while the remaining portion are fermented in the large intestine by microflora to produce short-chain fatty acids, which are absorbed and metabolized to produce energy.

Additionally, carbon dioxide plus some organic gases are also released, which together with short-chain fatty acids contribute to common gastrointestinal side effects such as bloating, nausea, flatulence and osmotic diarrhea associated with excessive sugar alcohol consumption (O'Donnell & Kearsley, 2012). However, the intensity of gastrointestinal side effects of sugar alcohols is different for each sugar alcohol and in different individuals, partly due to the different gastrointestinal tolerance of different people and the varying digestibility and absorption capacity of different sugar alcohols (livesey, 2003; O'Donnell & Kearsley, 2012) (**Table 1.2**).The Academy of Nutrition and Dietetics reported that up to 10 or 15 g/day of most sugar alcohols can be tolerated (Fitch and Keim, 2012). Furthermore, the organoleptic and physico-chemical properties also vary from one sugar alcohol to another. The chemical and metabolic properties of some commonly usedsugar alcohols compared to sucrose are discussed below and presented in **Table 1.2.**

i)   Xylitol

Xylitol is a five carbon crystalline sugar alcohol (**Fig. 1.7**) that is derived from the catalytic hydrogenation of its precursor, xylose (Zacharis, 2012). It however occurs naturally in numerous fruits and vegetables at levels less than 1% (Washuett *et al*., 1973) and also produced physiologically in human as part of hepatic carbohydrate metabolism at levels of about 5-15 g per day (Touster, 1974). Xylitol has about similar sweetness as sucrose (**Table 1.2**) and a taste characterized by a refreshing cooling sensation. Upon ingestion, about 50% is absorbed in the intestine (**Table 1.2**), while the remaining is fermented in the large intestine by colonic microflora to produce some gases and short chain fatty acids (Livesey, 2012). Absorbed xylitol is mostly metabolized in the liver to become part of normal carbohydrate and glucose metabolism via the glucoronic acid-pentose phosphate shunt of the pentose phosphate pathway (Touster, 1974), while less than 2% is excreted in the urine (Livesey, 2003). The relative glycemic response (RGR) and relative insulin response (RIR) of xylitol (compared to sucrose equivalent) are about 12 and 11 (sucrose: 68 and 45) respectively (**Table 1.2**).

Xylitol possesses a better gastrointestinal tolerance compared to somesugar alcohols like mannitol and lactitol due to its lower laxative threshold (**Table 1.2**) (Flambeau *et al*., 2012),

although this may vary among different individuals. Nevertheless, there are evidences that the gastrointestinal tolerance of xylitol may increase with regular consumption (Sakallioğlu *et al*., 2014). Xylitol has been approved by the US FDA as food additive for special dietary purposes and as a table top sweetener (Fitch and Keim, 2012). It is widely used in several products such as pharmaceuticals, chewing gum, oral mouth wash, candies confectionaries etc. Notable beneficial properties associated with xylitol consumption include low glycemic response (Livesey, 2012), prevention of dental caries (Mäkinen, 1991) and acute otitis media (Uhari *et al*., 1998) incidences.
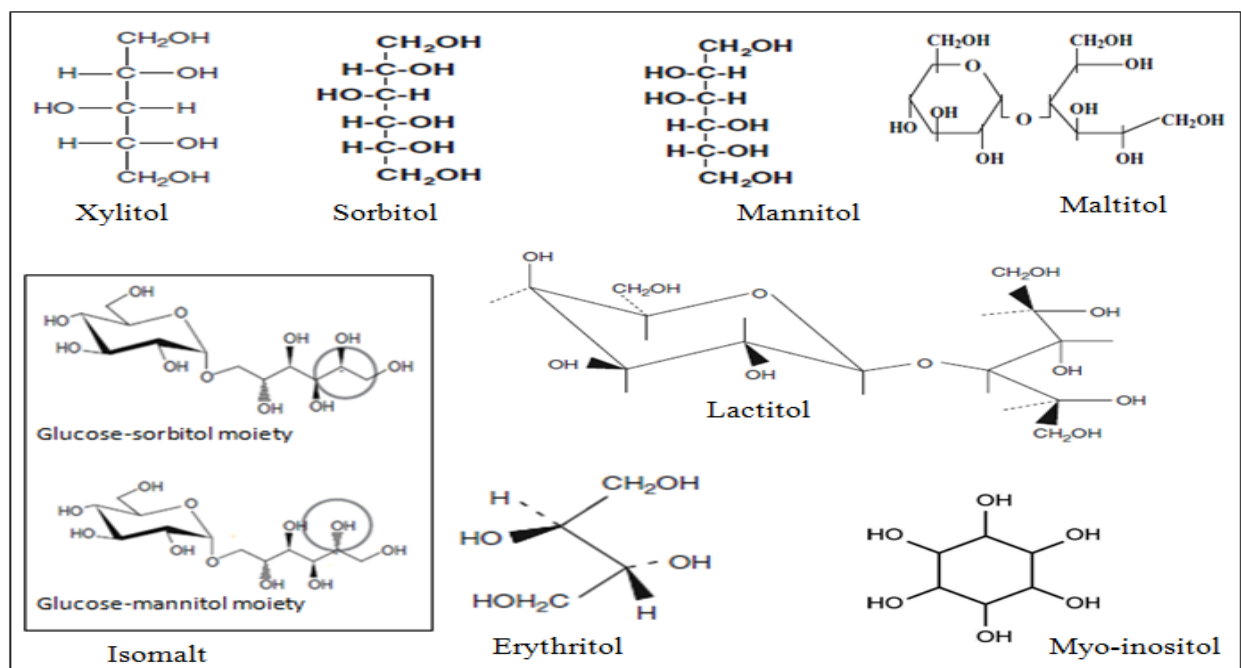
Figure 1.7: Chemical structures of some sugar alcohols (structures adapted without permission from O'Donnell & Kearsley, 2012).

ii)   Sorbitol and mannitol

Sorbitol and mannitol were the foremost sugar alcohols to be discovered and commercially made available. They are both white crystalline hexanols that share structural similarities except for the orientation of the hydroxyl group at carbon position "C2" (**Fig 1.7**) (Deis & Kearsley, 2012).

Sorbitol and mannitol occur naturally in appreciable amounts, more than most sugar alcohols. Sorbitol is found in a wide range of fruits and berries, while mannitol is found in most plants and sea weeds (Deis & Kearsley, 2012). Commercially, both sorbitol and mannitol are produced by the catalytic hydrogenation of simple reducing sugars. Hydrogenation of dextrose (aldose-sugar) yields sorbitol, while hydrogenation of fructose (ketose-sugar) derived from starch or sugar produces mannitol and sorbitol in varying proportions depending on the starting material (starch or sugar) (Deis & Kearsley, 2012).

Both sorbitol and mannitol are approximately 50% as sweet as sucrose and exhibit similar absorption capacities, which is about 25% of the amount ingested (**Table 1.2**).However, sorbitol is more extensively metabolized in the body than mannitol via carbohydrate metabolic pathways in the liver; hence, only negligible amounts ($< 2\%$) are excreted in the urine (Livesey, 2003). On the other hand, mannitol is almost completely excreted in the urine after absorption (Livesey, 2003). Accordingly, sorbitol has a RGR of 10, while mannitol has a RGR of 0 (**Table 1.2**).

Like most sugar alcohols, sorbitol and mannitol cause gastrointestinal side effects when consumed excessively, due to microfloral fermentation of unabsorbed portions in the large intestine. They are less tolerable compared to most sugar alcohols, but sorbitol is more tolerable than mannitol (**Table 1.2**). In fact, mannitol is known to be one of the least tolerated sugar alcohols. While 40 g of sorbitol per day may not cause any side effects, consumption of more than 20 g per day of mannitol can result in severe gastrointestinal symptoms (Deis & Kearsley, 2012). However, both sugar alcohols have been approved by US FDA as food additives, but with warnings on the risk of laxativeside effects (Fitch and Keim, 2012). Sorbitol and mannitol are used in several products, such as pharmaceuticals, candies, confectionaries, chewing gum etc, but sorbitol is more frequently and widely used, because it is more tolerable than mannitol. Other notable health benefits of sorbitol include prevention of caries incidence (Burt, 2006).

iii)   Maltitol

Maltitol powder is a white disaccharide sugar alcohol that is composed of glucose and sorbitol moiety (**Fig. 1.7**). It is commercially produced by the catalytic hydrogenation of maltose sugar.

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Maltitol syrup on the other hand is a hydrogenated starch hydrolysate, commercially produced from maize starch, maltose syrup or glucose syrup by catalytic hydrogenation (Flambeau *et al*., 2012). Maltitol syrup is predominantly composed of maltitol and small amounts of sorbitol and hydrogenated gluco-oligosaccharide. Maltitol is about 90% as sweet as sucrose (**Table 1.2**).

About 40% of ingested maltitol is absorbed in the intestine (Livesey, 2003). However, absorbed and unabsorbed maltitol can be hydrolyzed by physiological disaccharidase to give glucose and sorbitol (Lian-Loh *et al*., 1982; Zunft *et al*., 1983). Glucose is rapidly absorbed in the intestine, while sorbitol is passively absorbed and/or fermented (sorbitol) in the large intestine by colonic bacteria. Hence, sorbitol is found in the urine after maltitol consumption (Lian-Loh *et al*., 1982; Zunft *et al*., 1983). Additionally, the glucose resulting from maltitol hydrolysis majorly contributes to its higher RGR and RIR compared to most sugar alcohols (**Table 1.2**). Unabsorbed maltitol or sorbitol is responsible for gastrointestinal side effects due to colonic bacterial fermentation. However, maltitol is better tolerated compared to than most commonly used sugar alcohols (**Table 1.2**), which might be due to its higher molecular weight and glucose-sorbitol moiety composition (Flambeau *et al*., 2012). Maltitol has been determined to be safe for consumption by JECFA. In Europe however, there are specific legal requirements, which limits the use of maltitol to mostly confectionery, baked goods, ice cream, desserts and fruit preparations (Flambeau *et al*., 2012).

iv)   Isomalt

Isomalt is a white crystalline sugar alcohol that is composed of disaccharide sugar alcohols with glucose-mannitol and glucose-sorbitol moieties (**Fig. 1.7**). Commercially, isomalt is produced by catalytic hydrogenation of isomaltulose derived from bacterial fermentation of sucrose sugar (Sentko & Willibald-Ettle, 2012). Isomalt is about 40% as sweet as sucrose and poorly digested or absorbed in the intestine (about 10% of ingested amount) compared to most sugar alcohols (**Table 1.2**). Like maltitol, isomalt can be hydrolyzed by intestinal disaccharidases, but to a lesser degree (Nilsson and Jägerstad, 1987). Hydrolysis of isomalt produces glucose, which is rapidly absorbed, as well as mannitol and sorbitol, which are partly absorbed and partly fermented. However the RGR of isomalt is lower than maltitol (**Table 1.2**), which is due to the lower degree

of enzymatic hydrolysis. Furthermore, although isomalt has same molecular weight as maltitol, it is less tolerable than maltitol (**Table 1.2**), which is due to its lower degree of enzymatic hydrolysis compared to maltitol. Up to 50 g per day of isomalt in spread doses is tolerable in humans (Sentko & Willibald-Ettle, 2012). Isomalt has been approved by JECFA and US FDA for use as a table top sweetener and in several products like pharmaceuticals, candies, breakfast cereals, fruit spreads, baked products, chewing gums, chocolates etc (Sentko & Willibald-Ettle, 2012). Health-related beneficial effects associated with isomalt include, glycemic control (Livesey G, 2003) and oral health care benefits (Featherstone, 1994).

v)   Lactitol

Lactitol is a white crystalline disaccharide sugar alcohol that is composed of sorbitol and galactose moiety (**Fig. 1.7**). It is commercially produced by the catalytic hydrogenation of lactose (milk sugar) (van Velthuijsen, 1979). Lactitol is about 40% as sweet as sucrose andthe least absorbed polyol in the intestine (about 2% of the ingested amount) (**Table 1.2**). The remaining portion is fermented in the colon to produce some gases and short chain fatty acids. Studies have shown that lactitol is slowly hydrolyzed by intestinal disaccharidase (galactosidase) at a rate that is one-tenth the activity of galactosidase on lactose (van Velthuijsen, 1979). Due to the relatively slow absorption and metabolism of lactitol, it exhibits lesser RGR and RIR compared to most othersugar alcohols (**Table 1.2**). Lactitol possesses approximately similar laxative threshold as mannitol, which are both the least tolerated sugar alcohols (Flambeau *et al*., 2012) (**Table 1.2**). Studies have shown that consumption of 20 g lactitol caused gastrointestinal symptoms such as colic, flatulence, borborygmi, and bloating (Lee & Storey, 1999).Lactose mal-absorbers are more susceptible than normal lactose absorbers (Soontornchai *et al*., 1999). The use of lactitol has been approved by different regulatory bodies in different countries, but with warnings on its laxative effects. It is used in the production of pharmaceutical products, desserts, confectioneries, chewing gum, chocolates, baked products etc.

vi)   Erythritol (non-caloric sugar alcohol)

Erythritol is a white crystalline four carbon sugar alcohols (**Fig. 1.7**) that occurs in many fruits and vegetables. Commercially, erythritol is produced by yeast fermentation of a dextrose- or sucrose-rich solution (de Cock, 2012). It is about 70% as sweet as sucrose and also the most absorbed sugar alcohol in the intestine, which is about 90% of the amount ingested (**Table 1.2**). However, absorbed erythritol is not metabolized in the body but completely excreted unchanged in the urine (O'Donnell & Kearsley, 2012). Accordingly, it has no caloric, glycemic and insulinaemic effects, unlike other commonly used sugar alcohols (**Table 1.2**). Additionally, since erythritol is almost completely absorbed in the intestine, it rarely causes undesirable gastrointestinal side effects. It is better tolerated than other sugar alcohols (**Table 1.2**), even at ingested amounts that are two to four times higher than other sugar alcohols (de Cock, 2012). Erythritol has been endorsed by both JECFA and the US FDA as a safe general use sweetener (de Cock, 2012; Fitch and Keim, 2012). It is widely used as a table-top sweetener and in products like candies, bakery products, confectionaries, chewing gums, chocolates etc. Health benefits associated with erythritol include glycemic control, oral health care (Mäkinen *et al*., 2005) and anti-oxidant effects (den Hartog *et al*., 2010).

vii)   Myo-inositol

Myo-inositol is a white crystalline cyclic sugar alcohol that is an isomer of glucose (**Fig. 1.7**). It belongs to the group of stereoisomers collectively called inositol (1,2,3,4,5,6-cyclohexanehexol or simply cyclohexanehexol). However, myo-inositol is the main inositol stereoisomer in the human body (comprising over 90% of cellular inositol) and the most consumed or supplemented inositol stereoisomer (Chung & Kwon, 1999; McLaurin *et al*., 2000). Myo-inosiol alsooccurs naturally in several plants, mostly in citrusfruits and grains (Rex & Darnell, 1980; Lee & Coates, 2007). Commercially, it is produced from hydrolysis of crude phytate (US FDA, 2015). In human, myo-inositol is synthesized from glucose-6-phosphate by enzyme-catalyzed isomerization and dephosphorylation, which mostly occur in the kidney (Parthasarathy *et al*., 2006). However, dietary myo-inositol is obtained from myo-inositol supplement and phytic acid (inositol hexaphosphate), a storage form of myo-inositol in fruits and grains (Rex & Darnell,

1980). In the intestine, inositol hexaphosphateis hydrolyzed by phytase to release myo-inositol (Lam *et al*., 2006).

Myo-inositol is about 48 to 50% as sweet as sucrose and is absorbed in the intestine. Majority of ingested myo-inositol are absorbed in the intestine, while the remaining is fermented in the colon. Absorbed myo-inositol is used as a precursor for inositol phospholipid synthesis and as a part of cell membrane phospholipids, while excess inositol is metabolized via the glucuronic acid – pentose phosphate pathway (Lam *et al*., 2006). Myo-inositol has been reported to cause mild gastrointestinal symptoms, with 18 g per day being the maximum tolerable dose (Lam *et al*., 2006). Myo-inositol has been approved by the US FDA to be used as a food supplement and for special dietary purposes, including manufacturing of beverages, candies and infant milk (US FDA, 2015). Dietary supplementation of myo-inositol has been reported to show promising beneficial effects in the treatment of polycystic ovary syndrome (Ciotta *et al*., 2011), fertility and menstrual problems (Artini *et al*., 2013) as well as neurological disorders (Palatnik *et al*., 2001).

The lower glycemic response of sugar alcohols compared to sucrose (**Table 1.2**) is an important quality that makes them useful in glycemic control, especially in diabetics. However, advances in research have further suggested that sugar alcohols may also possess anti-hyperglycemic potential and may be useful in the management of diabetes and its related complications. In line with the aim of this study, the anti-hyperglycemic potencies of some commonly used sugar alcohol are discussed below.

1.2.11   Anti-hyperglycemic potentials of some commonly usedsugar alcohols

Xylitol is the most studied polyol that has shown promising anti-hyperglycemic and anti-diabetic potentials. Previous studies conducted by Islam and co-authors have consistently shown that 10% xylitol solution as a replacement of drinking water significantly reduced ($p< 0.05$) NFBG levels in normal ratsand also improved glucose toleranceand pancreatic beta-cell histology in diabetic rats (Islam, 2011, Islam & Indrajit, 2012; Rahman & Islam, 2014). In normoglycemic human subjects, 50 g xylitol in 300 mL of water supplied via nasogastric tube sparingly increased plasma glucose, but significantly increased GLP-1 hormone compared to glucose

administration (Woelnerhanssen *et al.,* 2016). Accordingly, it was concluded that xylitol may be useful in the control of postprandial blood glucose rise. Consistent with these data, Kang *et al*. (2014) and Chukwuma & Islam (2015) reported the significant *in vitro* inhibitory effects of xylitol on alpha glucosidase and alpha amylase activities, while other animal studies provided evidences on the inhibitory effect of xylitol on intestinal glucose absorption *in sit u*(Frejnagel *et al*., 2003) and in normal and diabetic animals (Chukwuma & Islam, 2015). Chukwuma & Islam (2015) later concluded that the inhibitory effect of xylitol on intestinal glucose absorption may be influenced by the delayed gastric emptying, concomitantly observed in the xylitol fed diabetic animals of their study (Chukwuma & Islam, 2015). Additionally the potency of xylitol to ameliorate insulin resistance has also been demonstrated in diabetic rats with NEFA-induced insulin resistance (Kishore *et al*., 2012), while data from other *ex vivo* and *in vivo* studies suggests the potency of xylitol to improve diabetes-induced insulin depletion (Rahman and Islam, 2014) and enhance insulin-mediated muscle glucose uptake (Chukwuma and Islam, 2015).

Maltitol is the most absorbed and metabolized disaccharide sugar alcohol (**Table 1.2**). Nevertheless, its effect on glucose and insulin response is lower than sucrose in both normal and non-insulin dependent diabetic patients. In normal subjects, a single oral dose of 50 g maltitol resulted in significantly lower glycemic and insulin responses compared to same amount of glucose or sucrose (Secchi *et al*., 1986; Matsuo, 2003). In non-insulin dependent diabetes subjects, single oral administration of 30 g or 50 g maltitol also exerted lower effects on glucose and insulin response compared to glucose or sucrose (Moon *et al*., 1990, Vessby *et al*., 1990). In fact, 50 g of maltitol caused a peak mean blood glucose that was only about 30% compared to 50 g glucose (Moon *et al*., 1990), which suggests the potency of maltitol in glycemic control during diabetes. Furthermore, the anti-hyperglycemic potential of maltitol has been demonstrated in overweight subject, who showed improved blood glucose, insulin and lipidemic response as well as increased satiety after consumption of maltitol-containing low calorie muffins compared to subjects fed with conventional sugar-sweetened muffins (Quilez *et al*., 2007). Data suggests that maltitol may be useful in the management of obesity-related diabetes. Additionally, *in vitro* studies showed that maltitol exhibited significant inhibition on alpha glucosidase and alpha amylase activities (Kang *et al*., 2014), thus may have clinical benefits to diabetics via reducing carbohydrate digestion, glucose absorption and postprandial hyperglycemia.

The *in vitro* and clinical anti-hyperglycemic potentials of myo-inostol have been demonstrated in a number of previous studies, when most data from different studies suggests the potency of myo-inositol supplement in improving insulin resistance and enhancing insulin-mediated peripheral glucose uptake. In humans, myo-inositol participates in glucose metabolism via its metabolites such as P-type inositol phosphoglycans (Saltiel, 1990; Shashkin *et al*., 1997). P-type inositol phosphoglycans are markers of insulin-mediated glucose uptake, which are involved in the upregulation of glycolysis via activation of pyruvate dehydrogenase complex (Kunjara *et al*., 1999; McLean *et al*., 2008). In previousstudies, it has been reported that both 6 months (Giordano *et al*., 2011) and 1 year (Santamaria *et al*., 2012) supplementation of 2000 mg myo-inositol (twice daily) was associated with the improvement of biomarkers of glucose metabolism and insulin sensitivity in postmenopausal women with metabolic syndrome. In another study, Dang *et al*. (2010) reported that oral administration of 1000 mg/kg myo-inositol facilitated cellular Glut-4 translocation 30 min after the ingestion in mice (Dang *et al*., 2010). In fact, 1 mM of myo-inositol and/or several inositol metabolites have been reported to be as effective as 100 nM insulin in stimulating Glut-4 translocation to the cell membrane *in vitro* (Yap *et al*., 2007), suggesting the potency of myo-inositol to improve insulin resistance and enhance peripheral insulin sensitivity. Accordingly, dietary myo-inositol has been shown to exert hypoglycemic effects in experimental animal (Dang *et al*., 2010) and human subjects with metabolic syndrome (Maeba *et al*., 2008).

Sorbitol is believed to be the most widely used polyol and several studies have demonstrated that sorbitol may be useful in glycemic control. Previous studies have reported the glycogenic effects of oral or parenteral sorbitol in rats (Stetten & Stetten, 1951), which suggests the possibility of sorbitol to enhance clearance of excess blood glucose towards glycogen synthesis. In humans, 35 g of orally administered sorbitol caused significantly lower mean peak increment of plasma glucose compared to sucrose or fructose in both normal and diabetic subjects (Akgün & Ertel, 1980). Furthermore, based on the data from several absorption studies, JECFA proposed that sorbitol may possess the potency to inhibit intestinal glucose absorption (World Health Organization, 1985), and consequently control postprandial blood glucose elevation. However, mechanism behind this effect of sorbitol is still not clear. Recently, it was reported that sorbitol plus some commonly used polyols exhibited appreciable inhibitory effects

on alpha glucosidase and alpha amylase activities *in vitro* (Kang *et al*., 2014), which further confirms the potency of sorbitol to control postprandial glucose elevation.

Several studies have demonstrated the glycemic control effect of erythritol. In a previous study, Bornet *et al*. (1996) reported that in healthy human subjects, the mean levels of plasma glucose and insulin remained unaffected up to 3 hours after a single oral administration of 1 g/kg bw of erythritol (Bornet *et al*., 1996). In diabetic rats, 10 days oral administration of 100 to 400 mg/kg bw of erythritol significantly reduced glucose level as well as oxidative stress markers like protein glycosylation and lipid peroxidation in the serum, liver and kidney (Yokozawa *et al*., 2002). Supporting clinical data in type 2 diabetic human subjects showed that 14 days oral administration of 20 g erythritol continuously reduced serum glucose and glycated hemoglobin (HbA1c), which suggests the potency of erythritol in ameliorating diabetes (Ishikawa *et al*., 1996). Furthermore, Flint *et al*. (2014) reported that short term (4 weeks) oral administration of erythritol (30 g/day) improved arterialendothelial function in T2D subjects (Flint *et al*., 2014), which suggests that erythritol may be useful in the management of diabetes-related microvascular complications. A most recent study showed that single dose of 75 g erythritol in 300 mL of water supplied via nasogastric tube modulated gut hormone (GPL-1) and also delayed gastric emptying in lean and obese normoglycemic subjects (Woelnerhanssen *et al*., 2016). Glucose level was also not significantly affected in this study, which suggests that erythritol could be useful in the control of postprandial glucose elevation and physiological glucose homeostasis.

1.2.12 Study rationale

Considering the above discussions, it is obvious to see that some commonly used sugar alcohols exhibit considerable glycemic control and anti-hyperglycemic potentials. The anti-hyperglycemic effects of xylitol and its potency in glycemic control via reducing intestinal glucose absorption and enhancing insulin-mediate glucose uptake have been widely investigated and demonstrated (refer to section 1.2.11). However, the possible anti-hyperglycemic mechanisms of action of other promising and commonly used sugar alcohols are still not clear.

1.2.13 Aim and objectives

The aim of this study was to further elucidate the possible sites and modes of action underlying the anti-hyperglycemic potentials of some selected commonly used sugar alcohols (maltitol, myo-inositol, sorbitol and erythritol). The research objectives included the following:

1. *Ex vivo* **study (experiment 1):**
   - Investigating the effects of increasing concentrations of selected sugar alcohols on glucose absorption in isolated rat small intestine.
   - Investigating the effects of increasing concentrations of selected polyols on glucose uptake in isolated rat psoas muscle.

2. *In vivo* **study (experiment 2 and 3):**
   - **Experiment 2:** Investigating the effects of a single bolusdose of selected sugar alcohols (maltitol, myo-inositol, sorbitol and erythritol) on gastric emptying, intestinal glucose absorption and digesta transit rate in both normal and type 2 diabetic rats.
   - **Experiment 3:** Investigating the molecular mechanisms behind the insulin-mediated muscle glucose uptake potential of erythritol in normal and type 2 diabetic rats.

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CHAPTER 2

MATERIALS AND METHODS

2.1   Materials

2.1.1   Chemicals and reagents

Streptozotocin, di-basic sodium phosphate, citric acid,sodium acetate,3-(N-morpholino) propanesulfonic acid (MOPS), acarbose, maltitol, sorbitol, adenosine 5′-triphosphate disodium salt hydrate (ATP), D-mannitol, phosphorus standard solution, 1st Strand cDNA Synthesis Kit for RT-PCR-AMV andammonium molybdatetetrahydrate were purchased from Sigma Aldrich, South Africa. 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, glucose-6-phosphate dehydrogenase, D-glucose 6-phosphate sodium salt, ferrous sulphateheptahydrate, trichloacetic acid (TCA), chloroform, sulphuric acid, isopropanol, ethanol, magnesium chloride, sodium hydroxide, disodium EDTA and myo-inositol were purchased from Merck, South Africa. Triethanolamine hydrochloride, diethylpyrocarbonate (DEPC), formamide, agarose, bromophenol blue, ethidum bromide and nicotinamide adenine dinucleotide phosphate ($NADP^+$) were purchased from Inqaba Biotech Company Ltd, South Africa. Glucose and fructose were purchased from Associated Chemical Enterprise (ACE), South Africa, while metformin and Novo rapid insulin were purchased from a local pharmacy store (Pharmed) in Durban, South Africa. Erythritol was purchased from Jungbunzlauer, South Africa.Insulin ELISA kit was purchased from Mercodia AB, Uppsala, Sweden. iTaq^{TM} Universal SYBR Green Supermix and 50x Tris/Acetic Acid/EDTA (TAE) buffer (pH, 8.0) were purchased from Bio-Rad Laboratories, Inc., South Africa, while 1kb Plus DNA ladder was purchased from Thermo Scientific, South Africa. CellTiter-Glo® Luminescent Cell Viability Assay kit was purchased from Promega Corporation, USA.

2.1.2   Equipments

Steri-Cult CO$_2$ incubator (Labotec, South Africa), Automated Chemistry Analyzer (LabmaxPlenno, Labtest Inc., Lagoa Santa, Brazil), Glucometer (Glucoplus Inc., Saint-Laurent, Quebec, Canada), Ultra Turrax Tube Drive Work Station Homogenizer (IKA-Works, Staufenim Breisgau, Germany), HettichMikro 200 Microcentrifuge (Hettich Lab Technology, Tuttlingen, Germany), Synergy HTX Multi-mode Reader (BioTek Instruments Inc, Winooski, USA), UV mini 1240 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan), IKA TP 18/10 Utra-Turrax Homogenizer (IKA-Works, Staufenim Breisgau, Germany), Microfuge 20R Centrifuge (Beckman Coulter, Inc., Germany), CFX96 Touch^{TM} Real-Time System (Bio-Rad Laboratories Inc., Hercules, Carlifonia), G:BOX F3 Gel Doc Imaging System (Syngen Laboratory Equipment Supplier, Cambridge, UK), NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, USA), Eppendorf™ 5810R Centrifuge (Fisher Scientific, UK) and Jenway PFP7 Flame Photometer (Jenway Ltd., Felsted, UK), Ecotron Incubation Shaker (Infrost HT, Switzerland).

2.2   Methods and experimental design
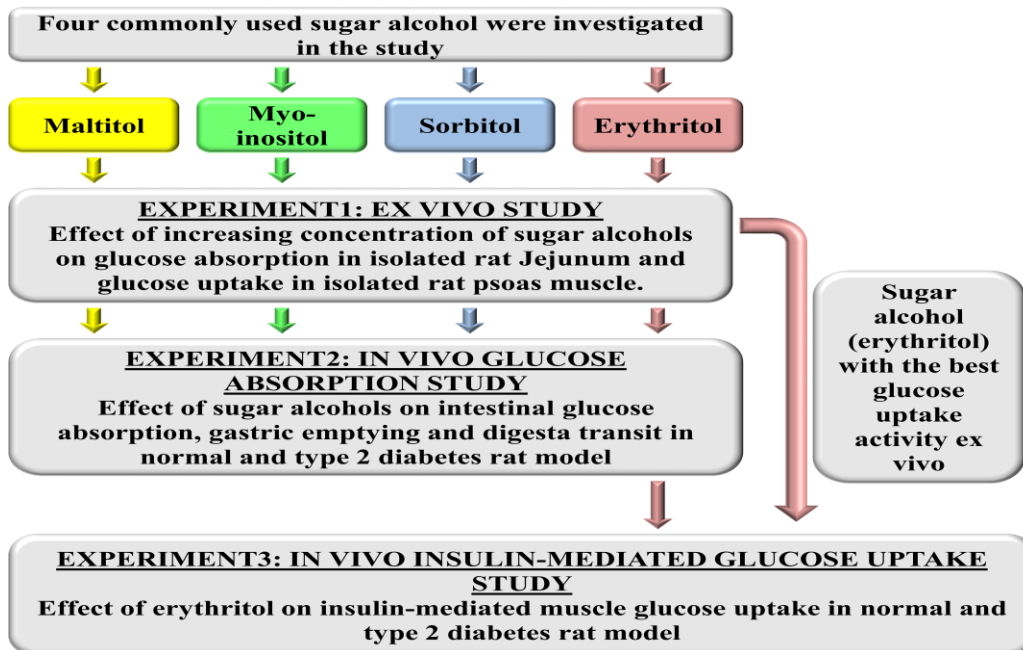
Figure 2.1: Experimental design of study

2.2.1   Experiment 1: *Ex vivo* study

Experiment 1 was conducted to determine the effect of increasing concentrations (2% -20%) maltitol, myo-inositol, sorbitol and erythritol on glucose absorption in isolated rat jejunum and glucose uptake in isolated rat psoas muscle. The schematic experimental design for this section of the study is presented in **Fig. 2.1**.

i)   Animals

Twenty adult male Sprague-Dawley (SD) rats(five rats for each sugar alcohol: maltitol, myo-inositol,sorbitol and erythritol)with mean body weight 183.20 ± 7.42 g were procured from the Biomedical Resource Unit (BRU) located at the Westville Campus of the University of KwaZulu-Natal, Durban, South Africa. The animals were fasted over-night (12 h) and euthanized by halothane anesthesia. Then the abdominal wall was dissected and the whole gastrointestinal tract (GIT) was collected immediately. Also the entire psoas muscle was collected immediately without causing any damage to the psoas muscle tissue. Intestines and psoas muscle tissues were immediately used for glucose absorption and glucose uptake study, respectively. All animal procedures were carried out according to the rules and regulations of the Animal Research Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa (Ethical approval number: 092/14/Animal).

ii)   Measurement of glucose absorption in isolated rat jejunum

This was measured by monitoring the time-dependent reduction of glucose concentration in an incubation solution containing 5 cm of freshly isolated rat jejunum and different concentrations (2.5% to 20%) of maltitol, myo-inositol, sorbitol and erythritol using methods reported previously (Hassan *et al*., 2010; Algandaby *et al*., 2010), with slight modification. Briefly, a 5 cm of jejunal segment from the isolated rat GIT was first inverted to expose the villi and then incubated (Steri-Cult CO_2 incubator, Labotec, South Africa) in 8 ml of Krebs buffer (118 mM NaCl, 5 mM KCl, 1.328 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 25 mM $NaHCO_3$) containing 11.1 mM (199.98 mg/dl) glucose and separately increasing concentrations

(2.5% to 20%) of maltitol, sorbitol, myo-inositol and erythritol. Incubation condition was at 5% CO_2, 95% oxygen and 37°C. Glucose with Krebs buffer, but without maltitol, myo-inositol, sorbitol and erythritol was used as a control for respective sugar alcohol. Glucose concentrations were measured by using an Automated Chemistry Analyzer (LabmaxPlenno, Labtest Inc., Lagoa Santa, Brazil) in all solutions before and after a 2 h incubation period. The intestinal glucose absorption was calculated as the amount of glucose (mg) absorbed per cm of rat jejunum using the following formula:

$$Intestinal\ glucose\ absorption\ (per\ cm\ of\ jejunum) = \frac{(GC1 - GC2)}{length\ of\ jejunum\ in\ cm}(Eq.\,2.1)$$

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

Percentage glucose absorption inhibition to determine the IC_{50} value was calculated as follows:

$$Inhibition(\%) = \frac{(\Delta GC\ for\ control\ - \Delta GC\ for\ sample)}{\Delta GC\ for\ control}\ x\ 100 \quad (Eq.\,2.2)$$

Where $\Delta GC = GC1 - GC2$.

iii)   Type of muscle tissue fiber and viability assay

Rat psoas muscle (hip flexor) is composed of type I and type II (IIA, IIX and IIB) muscle fibres, with more than 95% composition of type II, predominantly IIB (> 60%) (Eng *et al*., 2008), thus was suitable for this *ex vivo* study.The viability of isolated psoas muscle tissue during 1 hour incubation period was confirmed according to previously described methods (Best, 2008; Pichugin *et al*., 2006). These include estimating cellular ATP concentrations and cellular K^+/Na^+ ratio of freshly isolated muscle tissue and 1 hour after the incubation at rest in Kreb's buffer under similar experimental incubation conditions as used for *ex vivo* muscle glucose uptake study. ATP concentration (µM) was measured in tissue homogenate using CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega Corporation, Madison, USA). Briefly, a 0.5 g of muscle tissue was homogenized in 5 ml of ice cold PBS and centrifuged at 12,000 g for 5 min at 4°C. Thereafter, 90 µl of supernatant or ATP standard solution (3 to 30 µM) was mixed with 90

μl of ATP assay reagent and incubated for 10 min at 25°C. Luminescence was measured (Synergy HTX Multi-mode reader, BioTek Instruments Inc, Winooski, USA) and tissue ATP concentration (μM) was calculated from an ATP standard curve.

On the other hand cellular K^+/Na^+ ratio in muscle tissue was measured according to a previously reported method with slight modifications (Pichugin *et al*., 2006). Briefly, a 0.5 g of tissue (reduced into tiny portions of approximately 1 mm thick) was first washed twice in 10 ml of 0.3 M D-mannitol solution by a 2 min gentle agitation to remove ions from the extracellular spaces of the tiny slices. Thereafter, the washed tissue was incubated in 20 ml of 3% w/v trichloroacetic acid (TCA) for 24 h to rupture cells of tissue and release intracellular ions. The K^+ and Na^+ concentrations (ppm) were measured in the TCA supernatant using a flame photometer (Jenway PFP7 Flame Photometer, Jenway Ltd., Felsted, UK).

iv)   Measurement of glucose uptake in isolated rat psoas muscles

The effect of maltitol, myo-inositol, sorbitol and erythritol on glucose uptake in isolated rat psoas muscles was determined according to procedures modified from previously reported methods (Hassan *et al*. 2010; Algandaby *et al*. 2010), by monitoring the glucose concentration change in an incubation solution containing 0.5 g of freshly isolated rat psoas muscle and different concentrations (2.5% to 20%) of maltitol, myo-inositol, sorbitol and erythritol. Briefly, a 0.5 g of isolated rat psoas muscle was incubated in 8 ml of Krebs buffer containing 11.1 mM (199.98 mg/dl) glucose and increasing concentrations (2.5% to 20%) of maltitol, myo-inositol and erythritolor 2 mg/ml metformin (as positive control) with and without 200 mU/ml insulin. Incubation condition was at 5% CO_2, 95% oxygen and 37°C. Glucose with Krebs buffer, but without maltitol, myo-inositol, sorbitol and erythritol was used as a control. Glucose concentration was measured in all incubated solutions before and after 1 h incubation and the muscle glucose uptake was calculated as the amount of glucose (mg) absorbed per gram of rat psoas muscle using the following formula:

$$Muscle\ glucose\ uptake\ (per\ gram\ of\ muscle\ tissue)$$
$$= \frac{(GC1 - GC2)}{Amount\ of\ muscle\ tissue\ in\ gram} \qquad (Eq.\,2.3)$$

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

Percentage glucose uptake used to determine the concentration of sorbitol required to cause 50% increase of muscle glucose uptake (GU_{50}), with or without insulin was calculated as per following formula:

$$\% \; increase \; in \; glucose \; uptake = \frac{(\Delta GC \; for \; sample \; - \Delta GC \; for \; control)}{\Delta GC \; for \; control} \; x \; 100 \quad (Eq. \, 2.4)$$

Where $\Delta GC = GC1 - GC2$

2.2.2 Experiment 2: *In vivo* intestinal glucose absorption study

Experiment 2 was conducted to determine the acute effect of a bolus tolerable dose of maltitol, myo-inositol, sorbitol and erythritol on intestinal glucose absorption, gastric emptying, digesta transit and postprandial blood glucose increase in a normal and T2D rat model. The schematic experimental design for this section of the study is presented in **Fig. 2.2**.

i) Animals and grouping

Sixty-six seven weeks old male SD rats with mean body weight 182.32 ± 9.44 g were procured from the Biomedical Resource Unit located at the Westville Campus of the University of KwaZulu-Natal, Durban, South Africa. Animals were randomly divided into eleven (11) groups, with six animals in each group. The groups were namely: normal control (NC), normal maltitol (NML), normal myo-inositol (NMI), normal sorbitol (NSO), normal erythritol (NER), diabetic control (DBC), diabetic maltitol (DML), diabetic myo-inositol (DMI), diabetic sorbitol (DSO), diabetic erythritol (DER) and diabetic acarbose (DBA). 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: 092/14/Animal).
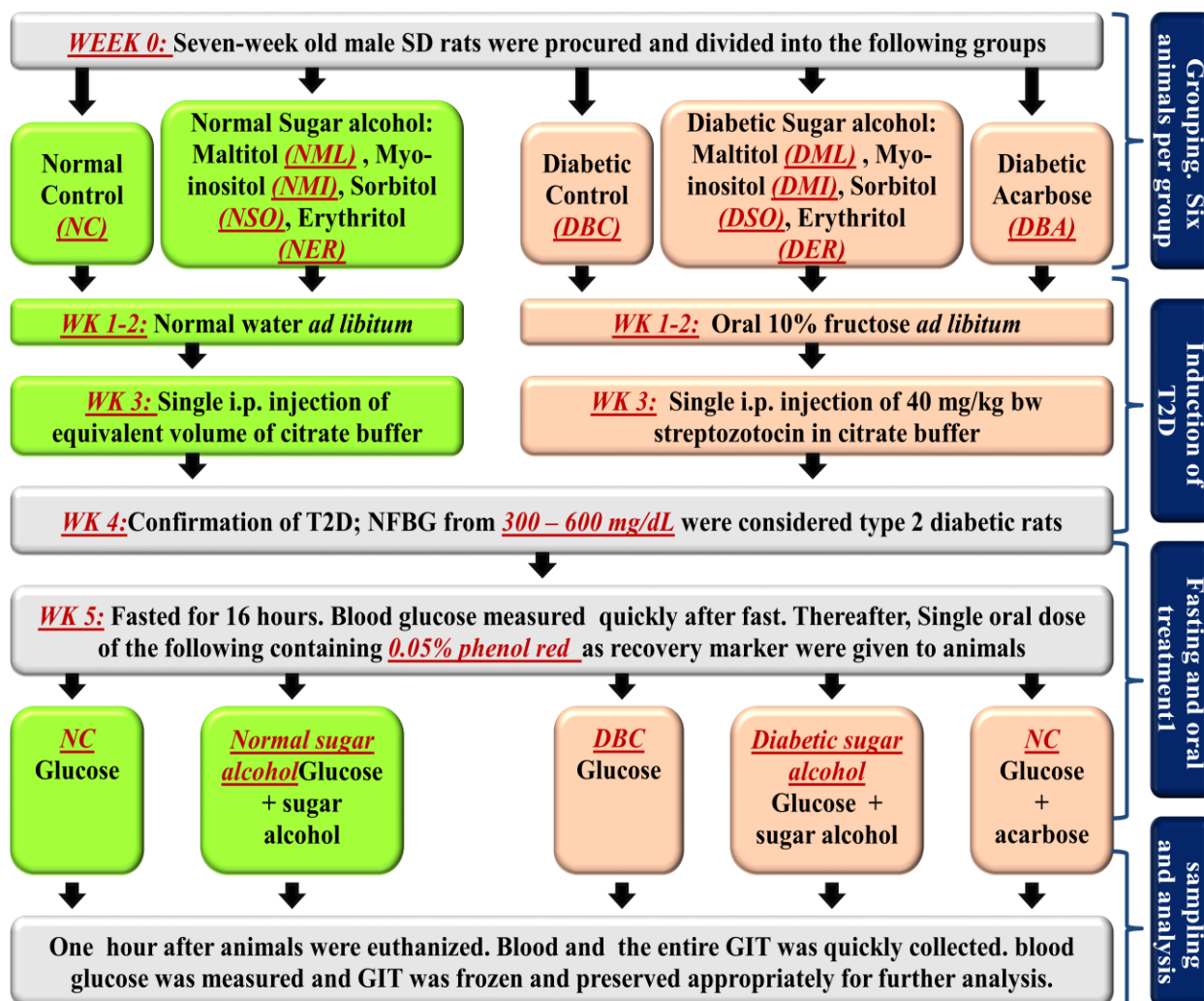
Figure 2.2: Experimental design of experiment 2 (*in vivo* intestinal glucose absorption study)

ii)   Induction of T2D

Insulin resistance and partial pancreatic β-cell dysfunction are the two major etiological and pathophysiological characteristics of T2D. In order to induce T2D, we adopted the methods reported previously (Wilson & Islam, 2012). Briefly, a 10% fructose solution was supplied in the form of drinking water for first two weeks to the animals in diabetic groups (DBC, DML, DMY, DSO, DER and DBA) to induce insulin resistance, while the animals in the non diabetic groups (NC, NML, NMY, NSO and NER) were supplied with normal drinking water. Thereafter, animals in diabetic groups were injected with a single dose (i.p.) of streptozotocin (40 mg/kg bw)

dissolved in citrate buffer (pH 4.5) to induce partial pancreatic β-cell dysfunction, whereas the animals in the non diabetic groups were injected with citrate buffer only. One week after the streptozotocin injection, non-fasting blood glucose levels of all animals was measured using a portable Glucometer (Glucoplus Inc., Saint-Laurent, Quebec, Canada), and rats with blood glucose level >300 mg/dl were considered as diabetic, and used for the study, while those with blood glucose levels < 300 mg/dl were excluded from the study.

iii)   Feeding and sampling

After the confirmation of diabetes, all animals were fasted overnight (16 h) with free access to drinking water only and then fasting blood glucose (mg/dl) was measured using a portable glucometer (Glucoplus Inc., Saint-Laurent, Quebec, Canada). Thereafter, animals in each group were orally administered the following as a bolus dose containing 0.05% phenol red (PR) as a recovery marker:

- Normal control (NC) group   : 2g/kg bw glucose
- Normal maltitol (NML) group   : 2 g/kg bw glucose + 0.6 g/kg bw maltitol
- Normal myo-inositol (NMI) group : 2 g/kg bw glucose + 1 g/kg bw myo-inositol
- Normal sorbitol (NSO) group   : 2 g/kg bw glucose +   0.4 g/kg bw sorbitol
- Normal erythritol (NER) group   : 2 g/kg bw glucose + 1 g/kg bw erythritol
- Diabetic control (DBC) group   : 2 g/kg bw glucose
- Diabetic maltitol (DML) group   : 2 g/kg bw glucose + 0.6 g/kg bw maltitol
- Diabetic myo-inositol (DMI) group : 2 g/kg bw glucose + 1 g/kg bw myo-inositol
- Diabetic sorbitol (DSO) group   : 2 g/kg bw glucose +   0.4 g/kg bw sorbitol
- Diabetic erythritol (DER) group : 2 g/kg bw glucose + 1 g/kg bw erythritol
- Diabetic acarbose (DBA) group   : 2 g/kg bw glucose +   100 g/kg bw acarbose

Oral bolus dose of sugar alcohols was chosen considering their gastrointestinal tolerance after ingestion (Livesey, 2003; Flambeau *et al.*, 2012).

Exactly 1h after the ingestion and without access to food or drinking water, animals were sacrificed using halothane anaesthesia and blood sample (using cardiac puncture) as well as the

entire GIT of each animal was quickly collected. Glucose concentration was measured in the blood samples using an Automated Chemistry Analyzer (LabmaxPlenno, Labtest, Lagoa Santa, Brazil). The GIT from each animal was frozen immediately in liquid nitrogen to prevent the movement of the contents, and then preserved immediately at -30°C for further analysis.

iv) Sample preparation and analysis

Each gastrointestinal tract (GIT) was thawed and divided into eight segments: stomach; 1st, 2nd, 3rd, and 4th quarters of small intestine; cecum; proximal and distal half of the colon. Content weight of each segment was determined by subtracting the weight of the segment without content from the respective weight of the segment with content. Contents and tissues were collected and individually homogenized in ice cold normal saline (Ultra Turrax Tube Drive Work Station homogenizer, IKA Laboratory equipment, Staufen, Germany) and centrifuged twice at 15,000 rpm for 30 min (HettichMikro 200 microcentrifuge, Hettich Lab Technology, Tuttlingen, Germany) as reported previously (Chukwuma& Islam, 2015). Phenol red (PR) concentration was determined spectrophotometrically (Synergy HTX Multi-mode reader, BioTek Instruments Inc, Winooski, USA) with bile acid correction in the supernatants of contents and tissue segments according to a previously published method with slight modifications (French *et al*., 1968). Briefly, 30 µl of supernatant or phenol red standard (concentrations 0.0038% - 0.00025%) was mixed with 210 µl of 0.1 M dibasic sodium phosphate solution (pH 10.5). Then the optical density at 420 nm was subtracted from the optical density at 620 nm (for bile acid correction) to obtain the final optical density. The concentration of PR was calculated from the standard curve. Glucose concentration in the intestinal contents was measured using an Automated Chemistry Analyzer (LabmaxPlenno, Labtest Inc., Lagoa Santa, Brazil) using commercial assay kits.

v) Calculations

Glucose (g) and PR (g) recovered from each GIT segment was calculated using the following formula:

$Glucose\ recovered\ (g)$

$$= \frac{Glucose\ Concentration\ \left(\frac{mg}{dl}\right)}{100\ 000} \times Homogenization\ volume\ (mL)\quad (Eq.\ 2.5)$$

$$PR\ recovered\ (g) = \frac{PR\ Concentration\ \left(\%\frac{w}{v}\right)}{100} \times Homogenization\ volume\ (mL)\ (Eq.\ 2.6)$$

Where, 1 mg/dl ≡ 0.00001 g/ml and 1% w/v ≡ 0.01 g/ml

The calculated glucose (g) and PR recovered (g) were used as indexes to calculate gastric emptying, glucose absorption index (GAI) and digesta transit according to previously described methods (Chukwuma& Islam, 2015).

Gastric emptying, denoting the degree of emptying of stomach content was calculated using the following formula:

$$Gastric\ emptying\ (\%) = \frac{A - B}{A} \times 100 \qquad (Eq.\ 2.7)$$

Where, "A" is the total amount of PR (g) recovered from GIT; and "B" is the total amount of PR (g) recovered from the stomach.

Glucose absorption index (GAI) denotes the degree of glucose absorption in each segment of GIT. It is the percentage amount of the glucose absorbed passing through a given segment of GIT, and was calculated using the following formula:

$$Glucose\ absorption\ index\ (\%)\ in\ a\ given\ segment\ of\ GIT = 1 - \frac{\frac{a}{b}}{\frac{c}{d}} \times 100 \qquad (Eq.\ 2.8)$$

Where, "a" is the amount of glucose (g) recovered from that segment; "b" is the amount of phenol red (g) recovered from the same segment; "c" is the amount of glucose (g) given to corresponding animal; and "d" is the amount of phenol red (g) given to the corresponding animal.

Digesta transit in a particular segment of the intestine is the ratio of the amount of content leaving that segment to the amount reaching the same segment. It was calculated in percentage using the following formula:

$$Digesta\ transit\ in\ a\ given\ segment\ (\%) = \frac{a}{b} \ X \ 100 \qquad (Eq.\ 2.9)$$

Where "a" is the 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 and "b" is the amount of phenol red (g) recovered from that particular segment of the GIT to the distal colon.

2.2.3 Experiment 3: *In vivo* muscle glucose uptake study

Experiment 3 was conducted to determine the acute effect of a bolus erythritol treatment on glucose tolerance, insulin secretion, liver and muscle gluconeogenic and glycolytic enzyme activities and mRNA expression of Glut-4 and IRS-1 in a normal and T2D rat models. The schematic experimental design for this section of the study is presented in **Fig. 2.3**.

i)   Animals and grouping

Sixteen seven weeks old male SD rats with mean body weight 191.93 ± 13.60 g were procured from the Biomedical Resource Unit located at the Westville Campus of the University of KwaZulu-Natal, Durban, South Africa. Animals were randomly divided into four groups, with four animals in each group. The groups were namely: normal control (NC), normal erythritol (NER), diabetic control (DBC) and diabetic erythritol (DER). 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: AREC/099/015D).
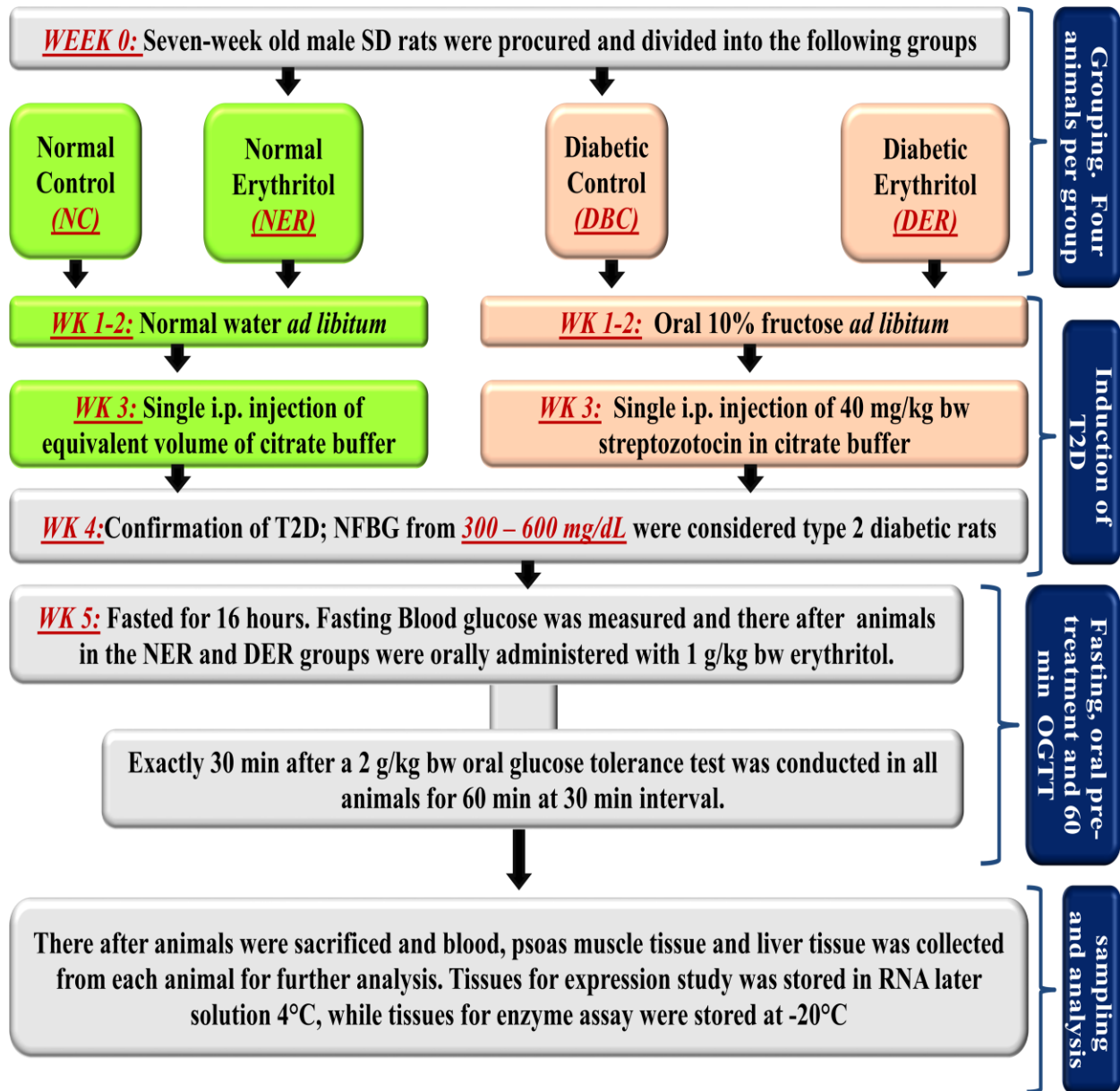
Figure 2.3: Experimental design ofexperiment 3 (*in vivo* muscle glucose uptake study)

ii)   Induction of T2D

Type 2 diabetes was induced and confirmed in the DBC and DER groups using similar methods described in chapter 2, part ii, sub-section 2.2.2, section 2.2, pages 70-71.

iii)   Animal treatments

A week after induction of diabetes, all animals were fasted overnight (16 h) with free access to drinking water only. After fasting, animals in the NER and DER groups were oral administered with 1 g/kg bwerythritol as a bolus dose. On the other hand, the animals in the NC and DBC groups were orally administered with equivalent volume of drinking water.

iv)   Oral glucose tolerance test (OGTT) and sampling

Exactly 30 min after oral erythritol dosing, a 1 h oral glucose tolerance test (OGTT) was carried out on all animals. To perform this test, a single dose of glucose solution (2 g/kg bw) was orally administered to each animal and thereafter blood glucose was measured at 0 (just before glucose ingestion), 30 and 60 min after the glucose ingestion using a portable glucometer (Glucoplus Inc., Saint-Laurent, Quebec, Canada). Immediately after OGGT, all animals were euthanized using halothane anesthesia. Blood was collected by cardiac puncture into sterile plain tubes to obtain the serum, while psoas muscle and liver tissues were collected and preserved at -30°C for further analysis. Muscle tissue for gene expression studies was preserved in RNA later at 2°C to 8°C, while the remaining liver and muscle tissues for enzyme assays were preserved at –30°C.

v)   Sample preparation

The blood was centrifuged (Eppendorf™ 5810R Centrifuge, Fisher Scientific, UK) at 3000 rpm for 15 min at 4°C to obtain the serum, which was preserved at –30°C for subsequent analysis. Liver and muscle tissue homogenates for enzyme assay were prepared according to a previously described method (Ngubane *et al*., 2011) with slight modifications. Briefly, a 0.5 g of tissue sample (liver or muscle) was homogenized (IKA TP 18/10 Utra-Turrax Homogenizer, IKA-Works, StaufenimBreisgau, Germany) in 5 ml of ice-cold homogenization buffer (50 mMTris-HCl buffer containing 100 mMKCl, 1 mM EDTA, pH 7.6). Homogenate was centrifuged (Microfuge 20R Centrifuge, Beckman Coulter, Inc., Germany) at 12,000 x g for 1 min at 4°C and supernatant was stored at -30°C for further analysis.

vi)   Measurement of serum insulin concentration

Serum insulin concentration was measured by an enzyme-linked immunosorbent assay (ELISA) method in a plate reader (Synergy HTX Multi-mode reader, BioTek Instruments Inc, Winooski, USA)using an ultrasensitive rat insulin ELISA kit (Mercodia, Uppsala, Sweden) according to the supplied protocols from the company. Briefly, 25 µl of serum or insulin standards (0.02, 0.05, 0.15, 0.40 and 1.00 µg/L) and 100 µl of 1X peroxidase-anti-insulin conjugate solution were measured into supplied mouse monoclonal anti-insulin coated wells. Mixture was incubated at 250 rpm (Ecotron Incubation Shaker, Infrost HT, Switzerland) for 3 h at room temperature. Thereafter wells were washed 6 times with 700 µl of a 1X wash buffer solution. A 200 µl of Tetramethylbenzidine (TMB) substrate was then added. Reaction was stopped by a 50 µl stop solution after 15 min of incubation at room temperature and absorbance was read at 450 nm in a plate reader (Synergy HTX Multi-mode reader, BioTek Instruments Inc, Winooski, USA). Serum insulin concentration was calculated from insulin standard curve and expressed in µIU/ml.

vii)   Measurement of protein concentration in tissue homogenates

The total protein concentration in tissue supernatant was measured using an automated chemistry analyzer (LabmaxPlenno, Labtest Co. Ltd., Lagoa Santa, Brazil) with commercial assaykits from the same company according to the biuret method of protein quantification.

viii)   Determination of liverglucokinase and muscle hexokinase activities

Hexokinase activity was measured by spectrophotometrically monitoring (340 nm) the $NADP^+$-dependent oxidation of glucose-6 phoshate (a product of hexokinase activity) according to a previously describe method (Bergmeyer *et al*., 1983) with slight modifications as described below.
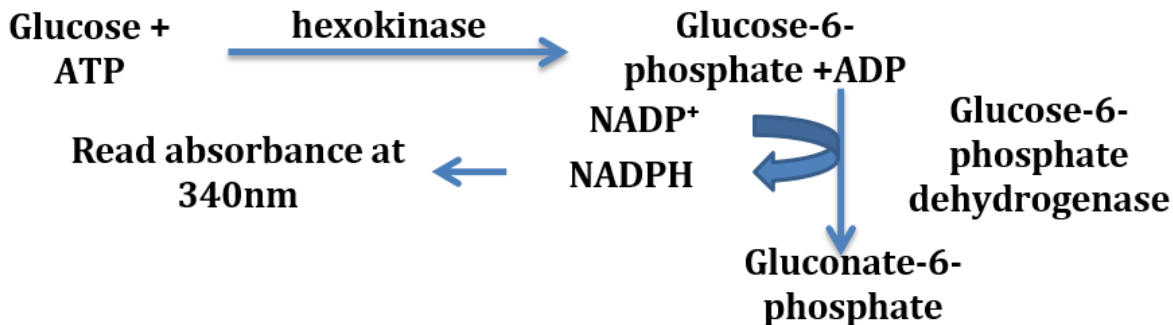
Figure 2.4: Schematic diagram showing the principle of tissue hexokinase assay

Briefly, a 0.5 ml of 50mMtriethanolamine buffer (pH 7.6), 0.5 ml of 555 mM D-glucose, 0.05 ml of 19 mM ATP, 0.1 ml of 100mM $MgCl_2$, 0.1 ml of 14mM $NADP^+$ and 0.02 ml of 125 U/ml glucose-6-phosphate dehydrogenase were mixed in a clean test tube and equilibrated to 25°C. Thereafter, a 0.025 ml of tissue homogenate (diluted to 15 mg/ml protein) or 0.025 ml of deionized water (for Blank) was added and immediately mixed by inversion. The increase in absorbance at 340 nm (UV mini 1240 spectrophotometer, Shimadzu Corporation, Kyoto, Japan) was immediately recorded for approximately 5 min and the reaction rate (ϕ) was calculated by dividing the increase in absorbance by the duration of time in minutes. The specific enzyme activity was calculated using the following formula:

$$Specific\ enzyme\ activity\ (Unit\ per\ mg\ protein) = \frac{(\phi_{test} - \phi_{blank})(RV)(DF)}{\varepsilon * SV * PC} \quad (Eq.\,2.10)$$

Where,

ϕ_{test} and ϕ_{blank}= Reaction rates of sample and blank respectively; RV = Reaction volume (ml); DF = Dilution factor; ε = 6.22, which is the millimolar extinction coefficient of NADPH at 340 nm; SV = Sample volume (ml); PC = 15 mg/ml, which is the protein concentration of sample used for assay.

One unit of enzyme will phosphorylate 1 μmole of D glucose per min at pH 7.6 at 25°C.

ix) Determination of liver glucose-6-phosphatase activity

Glucose-6-phospatase activity was determined by spectrophotometric quantification of glucose-6 phospatase-catalyzed inorganic phosphate (Pi) production from glucose-6-phospate substrate per unit time according to previously described methods (Taussky & Shorr, 1953; Nordlie & Arion, 1966) with slight modifications as described below.
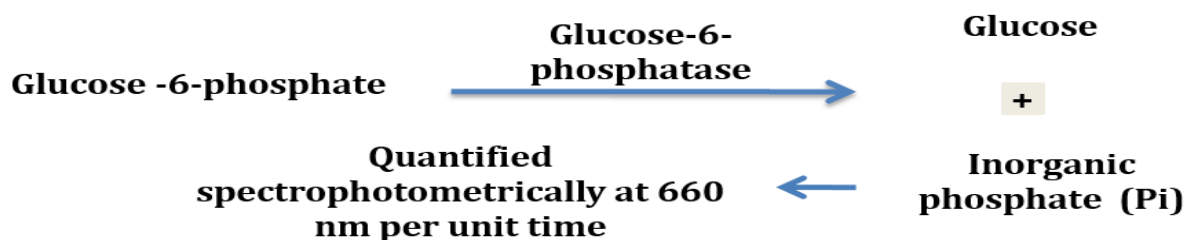
Figure 2.5: Schematic diagram showing the principle of tissue glucose-6 phosphataseassay

Briefly, a 0.15 ml of 100 mM bis-tris buffer (pH 6.5) and 0.05 ml of 200 mM glucose-6-phosphate solution were mixed in two separate microtubes, namely test and blank mixtures and then incubated for 5 min at 37°C. Thereafter, a 0.01 ml of liver homogenate (15 mg/ml protein) was first added to the test mixture. After 5 min incubation at 37°C, 0.045 ml of 20% TCA was added to both test and blank mixtures, followed by 0.01 ml of liver homogenate (15 mg/ml protein) to blank mixture only. Aliquots (0.125 ml) of supernatants (4000 rpm for 10 min) from both test and blank mixtures or phosphorus standard solutions (65, 130, 195, 260 and 325 µM) were then mixed with equal volume of Taussky-Shorr Colour Reagent (0.5 M sulfuric acid, 1% ammonium molybdatetetrahydrate and 5% ferrous sulfate heptahydrate) in a 96 well plate and incubated at 25°C for 5 min. The absorbance at 660 nm (Synergy HTX Multi-mode reader, BioTek Instruments Inc, Winooski, USA) of the blank mixture was first subtracted from the test mixture to eliminate the Pi(s) produced by other glucose-6-phophatase catalyzed reactions. The amount (µmole) of Pi librated form glucose-6phosphate was calculated from phosphorus standard curve. The specific activity of enzyme was calculated using the following formula:

$$Specific\ enzyme\ activty\ (Unit\ perm\ g\ protein) = \frac{(Pi\ in\ \mu mole)(RV)(DF)}{T * SV\ 1 * PC * SV\ 2} \quad (Eq.\ 2.11)$$

Where,

RV= Reaction volume (mL) of enzyme reaction; DF = Dilution factor; SV 1 = Sample volume (mL) of enzyme reaction; SV 2= Sample volume (mL) of Pi quantification reaction; PC = 15 mg/mL, which is the protein concentration of sample; T = Incubation time (min) of enzyme reaction.

One unit of enzyme activity $\equiv$ 1 μmol/min, which will liberate 1 μmole of Pi per min at pH 7.6 at 37°C.

x) Tissue RNA extraction

Total RNA in muscle tissue was extracted using the tri reagent extraction method according to a previously described protocol (Rio *et al*., 2010) with slight modifications. Briefly, muscle tissue (approximately 0.1 g) was homogenized (IKA TP 18/10 Utra-Turrax Homogenizer, IKA-Works, StaufenimBreisgau, Germany) in 1 ml of ice-cold TRIzol® buffer. Immediately, 0.2 ml of chloroform was added and mixture was vortexed briefly and allowed to stand for 10 min at 25°C before centrifuging at 12,000 x g for 15 min at 4°C. The aqueous phase of the supernatant was then transferred into a fresh tube and RNA was precipitated with 0.5 ml of isopropanol at room temperature for 10 min and thereafter pelleted at 12,000 x g for 10 min at 4°C (Microfuge 20R Centrifuge, Beckman Coulter, Inc., Germany). The RNA pellet was washed once with 1 ml of 75% ethanol at 7,500 x g for 5 min at 4°C, properly air-dried and then dissolved in 0.05 ml of ice-cold diethylpyrocarbonate (DEPC) treated water for immediate cDNA synthesis. RNA integrity was confirmed on a 2% agarose gel (containing 0.7 M formaldehyde) using autoclaved 1x MOPS running buffer (10x MOPS buffer: 0.2 M MOPS, 10 mM disodium EDTA and 50 mM sodium acetate, pH 7.0). Total RNA concentration and purity was determined using a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, Wilmington, USA).

xi)   cDNA synthesis from total RNA

Approximately 1 µg of total RNA was used for cDNA synthesis using a commercial cDNA synthesis kit (1st Strand cDNA Synthesis Kit for RT-PCR-AMV, Roche, Germany) according to protocols from the kit manufacturer. Reaction mixture contained 2 µL of reaction buffer (100 mM Tris-base and 500 mMKCl, pH 8.3), 4 µL of 25 mM $MgCl_2$, 2 µL of deoxynucleotide mix (contained 10 mM each of dATP, dCTP, dTTP, dGTP), 2 µL of 1.6 µg/µL p(dN)$_6$ Random primer, 1 µL of 50 U/µL RNase inhibitor, 1 µL of 16 U/µL Avian Myeloblastosis Virus (AMV) Reverse Transcriptase and varying volumes of RNase-free water and total RNA to make a final volume of 20 µL containing approximately 1 µg of total RNA. Mixture was vortexed and centrifuged briefly and thereafter incubated at room temperature for 10 min and at 42°C for 60 min consecutively. Mixture was then stored at -20°C and later used for Real-Time PCR (qPCR) (CFX96 Touch^{TM} Real-Time System, Bio-Rad Laboratories Inc., Hercules, Carlifonia).

xii)   Semi-quantitative determination of mRNA expression

Semi-quantitative determination of mRNA expression of target genes by Real-Time PCR (qPCR) was carried out in a CFX96 Touch^{TM} Real-Time System (Bio-Rad Laboratories Inc., Hercules, Carlifonia) using the iTaq^{TM} Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc., South Africa) and synthesized forward and reversed primers for both the genes of interest (Glut-4 and IRS-1 of the insulin signaling pathway) and reference gene (β-Actin) (**Table 2.1**) (Araujo   *et al*., 2004; Im *et al*., 2006) according to manufacturer's instructions. Reaction mixture contained 10 µl of supermix, 1 µl each of 0.5 µM forward and reverse primers and 1 µl 50 ng/µl cDNA for (for quantification) or 0.04 to 50 ng/µl cDNA (for determination of qPCR efficiency, also known as "E" value). The qPCR was carried out using the following "PrimePCR" program conditions: initial cDNA denaturation at 95°C for 2 min; 39 cycles (95°C for 5 sec denaturation and 54°C for 30 sec annealing); 95°C for 5 sec; and a melt curve from 65 to 95°C at 5°C increment per 5 sec. The computed efficiencies (E) (**Supplementary data 4**) (Pfaffl, 2001) and single melt peaks (**Supplementary data5**) were used as indexes for validating the quality of qPCR. The qPCR efficiency (E) was calculated from a qPCR standard curve (**Supplementary data 4**) using the following formula (Pfaffl, 2001).

$$E = 10^{[-1/slope]} \qquad (Eq.\ 2.12)$$

$$\% E = (E - 1) \times 100 \qquad (Eq.\ 2.13)$$

Where, E = qPCR efficiency and   % E = qPCR percentage efficiency.

Table 2.1: Information on gene, primers and expected product/amplicon size

Target gene	Accession number	Forward primer	Reverse primer	Expected product/amplicon size*
β-Actin	NM_031144.3	5'- ATG AAG ATC CTG ACC GAG CGT G-3'	5'-CTT GCT GAT CCA CAT CTG CTG G-3'	510 bp
Glut-4	NM_012751.1	5'-AGA GTC TAA AGC GCC T-3'	5'-CCG AGA CCA ACG TGA A-3'	297 bp
IRS-1	NM_012969.1	5'- ACC CAC TCC TAT CCC G-3'	5'-CCC TAC TCC GTT TGT CC-3'	375 bp

* Expected product/amplicon sizes and primer specificity of qPCR were determined *in silico* using SnapGene 3.2.1, windows version software (**Supplementary data 1, 2 & 3**).

The mRNA expression of the genes of interest was first normalized against the reference gene and thereafter the expression of the treated groups was expressed as fold change from their respective control groups using the following previously reported formulae (Schmittgen & Livak, 2008).

$$Fold\ change\ due\ to\ treatment\ = \frac{2^{-(\Delta Cq)}\ of\ treated\ group}{2^{-(\Delta Cq)}\ of\ control\ group} \qquad (Eq.\ 2.14)$$

Where,

Cq = Quantitation Cycle, which is the number of cycles required for the fluorescent signal from starting cDNA to be detected or exceeds background level during a qPCR assay.

ΔCq = Cq of gene of interest – Cq of reference gene.

The qPCR products for both the genes of interest (Glut-4 and IRS-1) and reference gene (β-Actin) were subjected to a 2% agarose gel electrophoresis at 60 volts for 2 h and then visualized by ethidium bromide staining in a G:BOX F3 gel doc imaging system (Syngen Laboratory equipment supplier, Cambridge, UK). Product sizes were estimated from a 1 kb Plus DNA ladder.

2.3 Statistical analysis

Data are presented as mean ± SD of either 3–5 replicates of determination for *ex vivo* study (**experiment 1**); 5-6 animals for *in vivo* intestinal glucose absorption study (**experiment 2**); and 4 animals for *in vivo* muscle glucose uptake study (**experiment 3**). All data were analyzed with a statistical software package (IBM SPSS, version 21 and 23) using Tukey's HSD multiple range post-hoc test. Results were considered significantly different at the value of $p < 0.05$.

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CHAPTER 3

MALTITOL INHIBITS SMALL INTESTINAL GLUCOSEABSORPTION AND INCREASES INSULIN MEDIATED MUSCLEGLUCOSE UPTAKE *EX VIVO* BUT NOT IN NORMAL AND TYPE2 DIABETIC RATS

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3.1 Abstract

This study investigated the effects of maltitol on intestinal glucose absorption and muscle glucose uptake using *ex vivo* and *in vivo* experimental models. The *ex vivo* experiment was conducted in isolated jejunum and psoas muscle from normal rats. The *in vivo* study investigated the effects of a single bolus dose of maltitol on gastric emptying, intestinal glucose absorption and digesta transit in normal and type 2 diabetic rats. Maltitol inhibited glucose absorption in isolated rat jejunum and increased glucose uptake in isolated rat psoas muscle in the presence of insulin but not in the absence of insulin. In contrast, maltitol did not significantly ($p>0.05$) alter small intestinal glucose absorption or blood glucose levels as well as gastric emptying and digesta transit in normal or type 2 diabetic rats. The results suggest that maltitol may not be a suitable dietary supplement for anti-diabetic food and food products to improve glycemic control.

3.2 Introduction

Global estimation of diabetes mellitus has revealed that approximately 415 million people were affected with the disease in 2015, and by 2040, this figure would become 642 million (International Diabetes Federation, 2015). Type 2 diabetes (T2D) is the most common form of diabetes mellitus. It is characterized by insulin resistance and pancreatic beta-cell failure, which leads to decrease glucose transport into the muscle and fat cells as well as increased gluconeogenesis and endogenous glucose production in the liver, causing hyperglycemia (Olokoba *et al*., 2012). T2D is a result of complex interaction between several factors such as genetic, environmental and behavioral risk factors (Olokoba *et al*., 2012; Wu *et al*., 2014). However, the most influential factors are the behavioral risk factors such as sedentary life style, consumption of high fat and high calorie diets as well as refined and simple carbohydrates like sucrose and fructose among others (Thorburn *et al*., 1989; Elliott *et al*., 2002; Astrup *et al*., 2002; Hu, 2003; Stanhope *et al*., 2011). Thorburn *et al*. (1989) reported that normal rats that consumed diet containing 35% of energy as fructose displayed reduced glucose disposal as well as reduced action of insulin in the liver and most peripheral tissue. Additionally, Stanhope *et al*. (2011) reported that postprandial triglycerides, LDL-cholesterol, and apolipoprotein-B in young male

and female adults were markedly increased after 12 days consumption of fructose and high fructose corn syrup that was equivalent to 25% of total dietary energy. Furthermore, in human subjects, 10 weeks consumption of sucrose equivalent to 28% of dietary energy increased food intake, body weight, body fat mass, and blood pressure compared to non-sucrose sweetener (Astrup *et al*. 2002). Hence, there has been a growing acceptance of the use of sugar substitutes as alternative sweetening agents in both non-diabetic and diabetic diets.

Sugar alcohol is one of the classes of sugar substitute that have gained popularity, especially for overweight, obese and diabetic individuals due to their lower caloric value and glycemic response compared to glucose and sucrose as well as minimal or no side effects (Livesey 2003, 2012; Islam & Indarjit, 2012; Fitch & Keim, 2012). Maltitol is one of the few commonly used disaccharide sugar alcohol formed from the catalytic hydrogenation of maltose to produce an alpha-l, 4 glucose-sorbitol linked polyol (Kearsley & Deis, 2012). Like most sugar alcohols, maltitol is poorly absorbed in the small intestine and also has a lower glycemic (35 versus 68) and insulinemic index (27 versus 45), lower caloric value (2.4 versus 4 kcal/g) and sweetening strength (about 90%) compared to sucrose (Kearsley & Deis, 2012; Livesey, 2012).

The oral health benefit of maltitol has been recognized in several *in vitro* and animal studies (Izumitani *et al*., 1989; Ooshima *et al*., 1992) as well as clinical trials (Li *et al*., 2010; Keukenmeester *et al*., 2014). However, the health benefit of maltitol is not limited to dental protection. Data from previous studies suggest that maltitol may also possess appreciable anti-hyperglycemic potentials (Matsuo, 2003; Quílez *et al*., 2007). This is because a single oral administration of 50 g maltitol in healthy subjects resulted in significantly lower glycemic and insulin responses compared to the administration of same amount of glucose or sucrose (Secchi *et al*., 1986; Matsuo, 2003). It has been also reported that the consumption of 30 or 50 g maltitol as a single oral dose resulted in lower glucose and insulin response compared to the consumption of equivalent amount of maltose or glucose by diabetic subjects (Mimura *et al*., 1972; Vessby *et al*., 1990). Furthermore, overweight subjects fed with maltitol-sweetened muffins containing low fat, low calories and high-amylose cornstarch showed reduced blood glucose level, insulin and lipidemic responses, but increased satiety compared to those fed with conventional sugar-sweetened muffin (Quílez *et al*., 2007). Additionally, two previous studies reported that a single oral administration of maltitol: sorbitol mixture (60:7) (Wheeler *et al*., 1990) or 50 g of maltitol

(Moon *et al*., 1990) caused significantly lower blood glucose and insulin responses compared to the administration of same amount of glucose in both normal and diabetic subjects.

In a more recent study, Kang *et al*. (2014) reported the *in vitro* alpha glucosidase, alpha amylase, and sucrase inhibitory activities of maltitol, which suggests that maltitol may be useful in controlling carbohydrate digestion and postprandial hyperglycemia. However, contrary to this, Matsuo (2003) reported that maltitol does not inhibit intestinal alpha glucosidase, sucrase, or maltase activities when a single oral dose of a mixture of maltitol and sucrose (25:25 g) or sucrose (50 g) or maltitol (50 g) was given to healthy human subjects. Based on the results of above-mentioned studies, we hypothesized that maltitol may show hypoglycemic response via other mechanisms such as inhibiting intestinal glucose absorption and/or increasing muscle glucose uptake rather than inhibiting intestinal carbohydrate digesting enzyme activities.

Therefore, this study was conducted to examine whether maltitol causes anti-hyperglycemic effects via inhibition of intestinal glucose absorption and/or increasing muscle glucose uptake by using *ex vivo* experimental model as well as normal and T2D models of rats.

3.3   Materials and methods

Please refer to chapter 2, sub-sections 2.2.1 and2.2.2, pages 66-74 for detailed material and methods of this article.

3.4   Results

3.4.1 *Ex vivo* study (experiment 1)

Effects of maltiol on intestinal glucose absorption and muscle glucose uptake

Data for the effects of maltiol on intestinal glucose absorption and muscle glucose uptake are presented in **Fig. 3.1** and **3.2** as well as **Table 3.1**. Incubation of jejunal tissue with all the tested concentrations of maltitol significantly inhibited glucose absorption ($p<0.05$) in a concentration-

dependent manner compared to the control (**Fig. 3.1**). The computed IC_{50} value for inhibiting glucose absorption by maltitol was $3.57 \pm 1.18\%$.
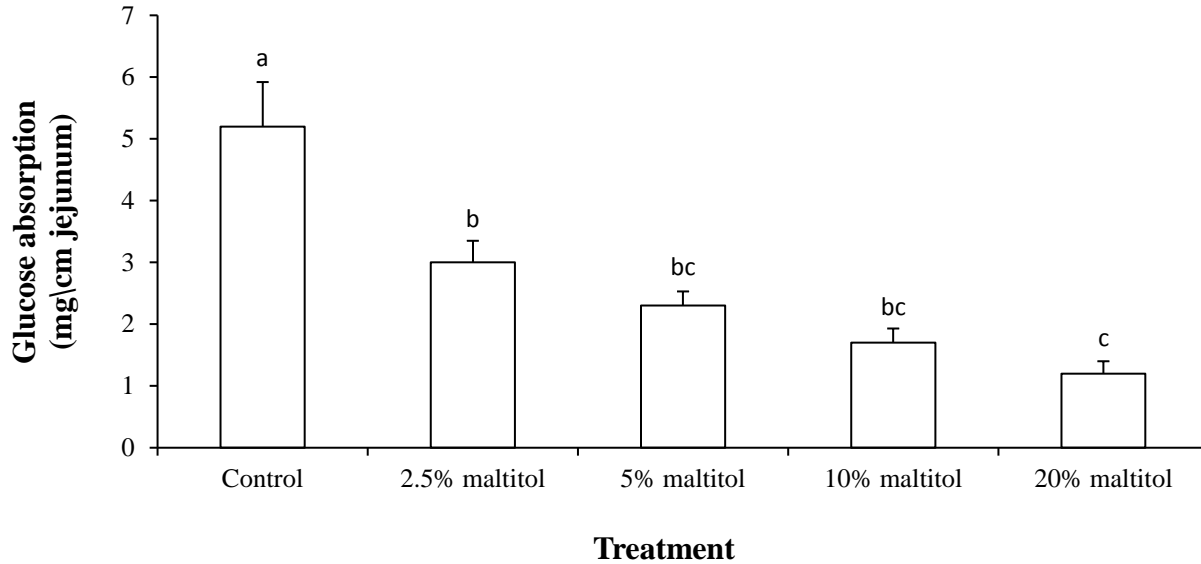
Figure 3.1: Effect of maltitol on glucose absorption in isolated rat jejunum. Data are presented as mean $\pm$ SD of triplicate analysis. [abc]Different letters presented above the bars are significantly different from each other (p<0.05; Tukey's HSD post-hoc test, IBM, SPSS version 21).

On the other hand, in the presence of insulin, maltitol increased glucose uptake in the isolated rat psoas muscle in a concentration dependent manner (**Fig. 3.2**). The glucose uptake at 20% maltitol concentration was significantly higher (p<0.05) than the control, but significantly lower (p<0.05) than a 2 mg/mL metformin containing sample, while glucose uptake at 2.5, 5 and 10% maltitol concentrations did not show significant differences compared to either the control or a 20% maltitol containing sample (**Fig. 3.2**).The computed GU_{50} value of maltitol was $7.31 \pm 2.08\%$ when treated with insulin (**Table 3.1**). However, in the absence of insulin, glucose uptake at all maltitol concentrations was significantly lower (p<0.05) than a 2 mg/mL metformin containing sample, but did not show any significant difference compared to the control (**Fig. 3.2**). The GU_{50} value was $111.12 \pm 19.36\%$ (**Table 3.1**), which is significantly higher than the GU_{50} value with insulin ($7.31 \pm 2.08\%$).
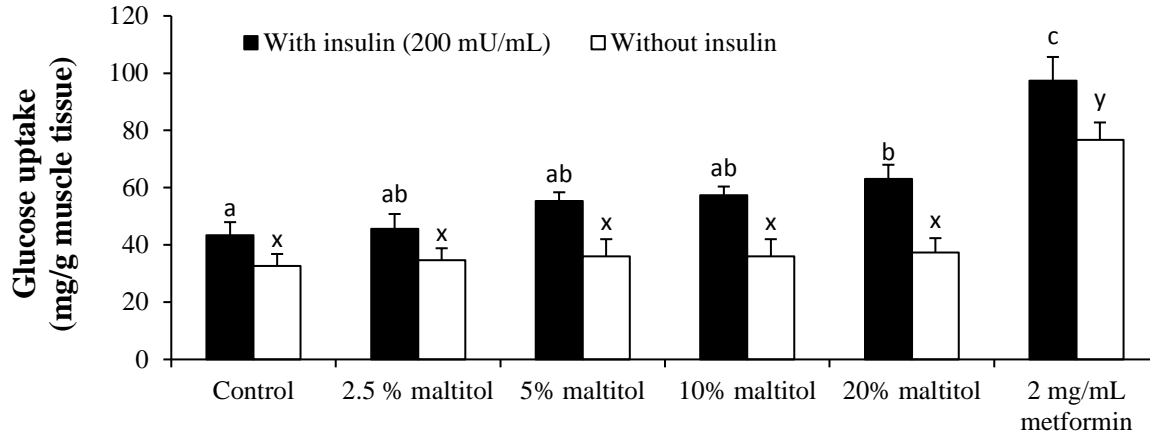
Figure 3.2: Effect of maltitol on glucose uptake with or without insulin in isolated rat psoas muscle. Data are presented as mean ± SD of triplicate analysis. [abc & xy]Different letters presented above the bars for with or without insulin are significantly different from each other (p<0.05; Tukey's HSD post-hoc test, IBM, SPSS version 21).

Table 3.1: GU_{50} values of percentage glucose uptake increase of maltitol in isolated psoas muscle, with and without insulin

	With insulin (200mU/mL)	Without insulin
GU_{50} (%)	7.31 ± 2.08^a	111.12 ± 19.36^b

Data are presented as mean ± SD of triplicates of analysis. [ab]Values with different superscript letters are significantly different from each other (p < 0.05; T-test, IBM, SPSS, version 21).GU_{50}: concentration (%w/v) of maltitol required to cause 50% increase of muscle glucose uptake.

Furthermore, 1 h incubation of psoas muscle tissue did not significantly affect the cellular ATP concentration and K^+/ Na^+ ratio compared to fresh tissue which was used for the muscle glucose uptake study (**Table 3.2**).

Table 3.2: Verification of psoas muscle tissue viability after 1 h of incubation in *ex vivo* muscle glucose uptake study

	Psoas muscle tissue viability indexes			
	ATP level (μM)	K^+/Na^+ ratio viability index		
		K^+ conc. (ppm)	Na^+ conc. (ppm)	K^+/Na^+ ratio
Freshly isolated tissue	21.27 ± 2.46	10.27 ± 0.30	1.11 ± 0.02	9.24 ± 0.30
After 1h incubation in Kreb's solution**	18.92 ± 2.63	9.53 ± 0.46	1.18 ± 0.10	8.12 ± 0.99

**Tissue incubation condition was similar to *ex vivo* experimental condition (5% CO_2, 95% oxygen and 37 °C). ppm: concentration in part per million.

3.4.2. *In vivo* intestinal glucose absorption study (experiment 2)

Effects of maltitol on gastric emptying, glucose absorption index, digesta transit and blood glucose level

The data for the effects of maltitol on gastric emptying, glucose absorption index and blood glucose level are presented in **Fig. 3.3** to **3.5** and **Table 3.3**. Gastric emptying was significantly increased ($p<0.05$) after induction of diabetes. Treatment with maltitol did not significantly affect the gastric emptying in normal and diabetic animals, while acarbose treatments significantly reduced ($p<0.05$) gastric emptying in diabetic animals (**Fig. 3.3**).
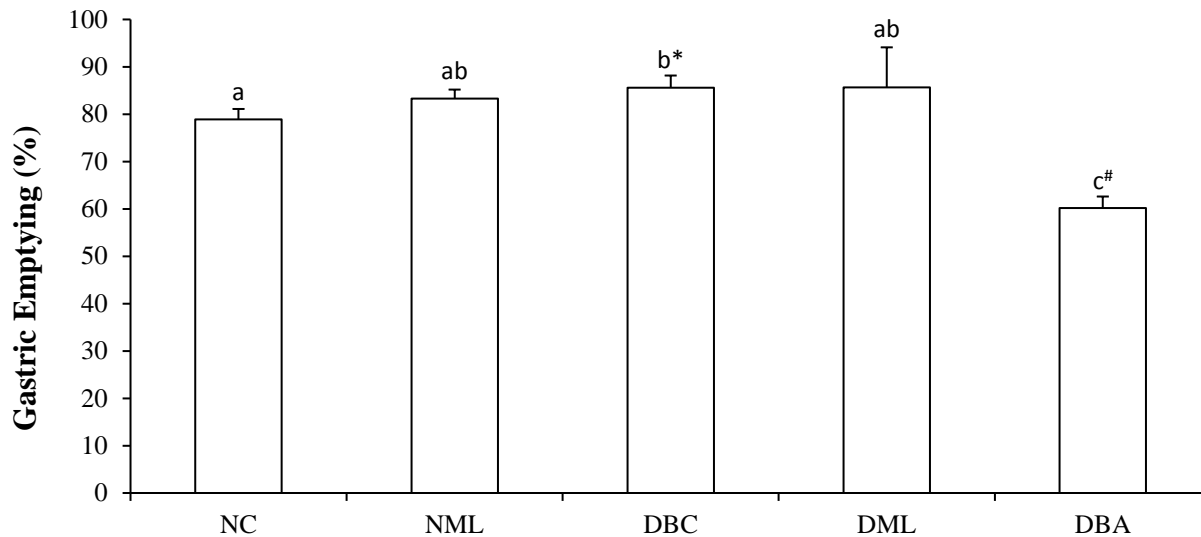
Figure 3.3: Effects of maltitol on gastric emptying in different animal groups at the end of a 1 h experimental period. Data are presented as mean ± SD of 5–6 animals. [abc]Different letters presented above the bars are significantly different from each other group of animals, *significantly different from NC group and [#]significantly different from DBC group (p<0.05; Tukey's HSD post-hoc test, IBM, SPSS version 21). NC: normal control; NML: normal maltitol; DBC: diabetic control; DML: diabetic maltitol; DBA: diabetic acarbose.

Furthermore, administration of maltitol did not significantly (p>0.05) affect the glucose absorption index GAI in the different intestinal segments of both normal and diabetic animals, while ingestion of acarbose significantly reduced (p<0.05) GAI in the 1st qtr of the small intestine, cecum and proximal colon of diabetic animals (**Fig. 3.4**). Additionally, ingestion of maltitol did not significantly lower postprandial blood glucose increase in both normal and diabetic animals, while ingestion of acarbose significantly (p<0.05) decreased postprandial blood glucose increase in diabetic animals (**Fig. 3.5**).
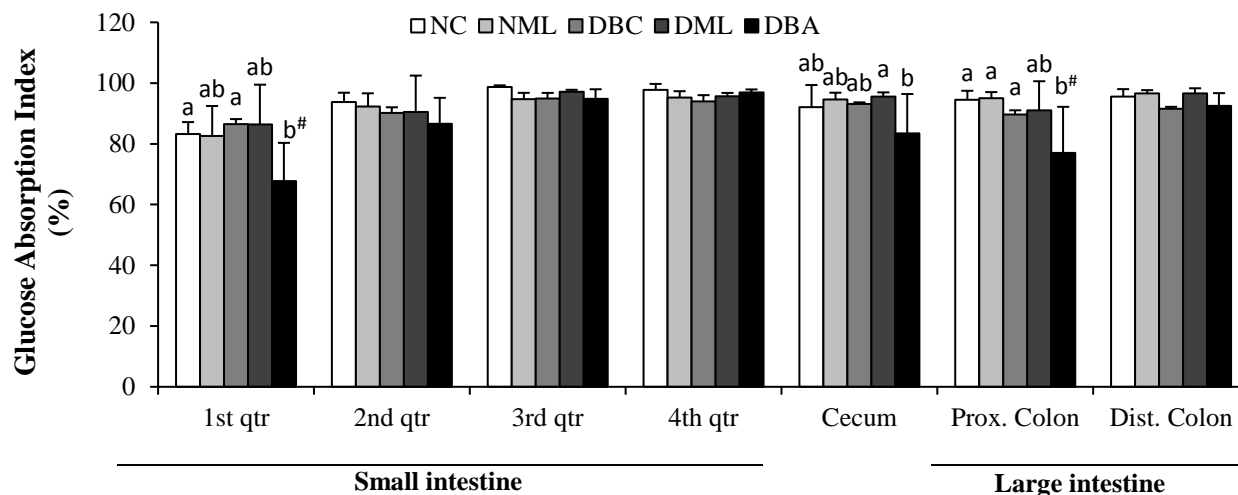
Figure 3.4: Effect of maltitol on glucose absorption index (GAI) in the different GIT segments of different animal groups at the end of a 1 h experimental period. Data are presented as mean ± SD of five to six animals. [ab]Different letters presented above the bars for a given segment are significantly different from each other group of animals, *significantly different from NC group and #significantly different from DBC group ($p<0.05$; Tukey's HSD post-hoc test, IBM, SPSS version 21). NC: normal control; NML: normal maltitol; DBC: diabetic control; DML: diabetic maltitol; DBA: diabetic acarbose.
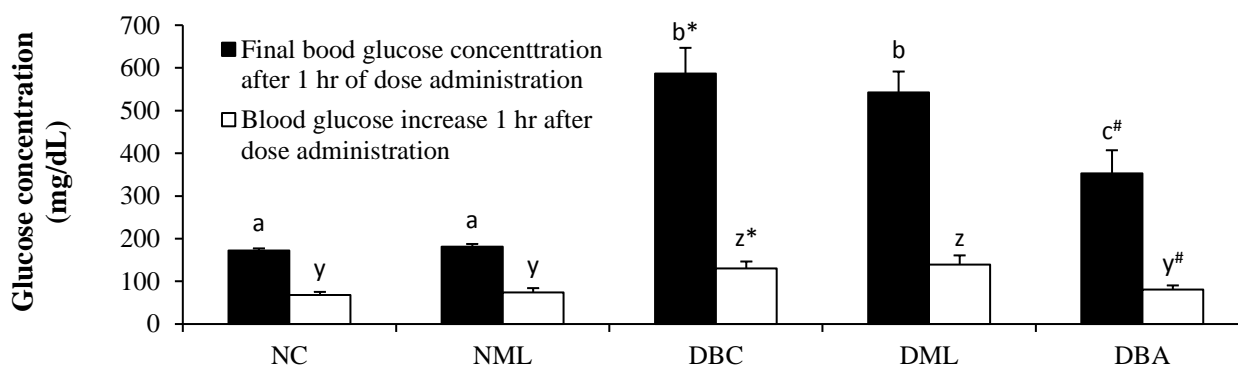
Figure 3.5: Effects of maltitol on final blood glucose concentration and blood glucose increase in animals after 1 h of the dose administration. Data are presented as mean ± SD of six animals. [abc& yz]Different letters presented above the bars are significantly different from each other group of animals, *significantly different from NC group and #significantly different from DBC group ($p<0.05$; Tukey's HSD multiple range post-hoc test, IBM, SPSS version 21).

The data for the effects of maltitol on intestinal digesta transit also revealed that maltitol or acarbose treatment did not significantly affect digesta transit percentage in the 1st to 3rd qtrs of the small intestine in normal or diabetic animals (**Table 3.3**). However, from the 4th qtr of the small intestine to the proximal colon, maltitol treatment significantly increased ($p<0.05$) digesta transit in normal animals. In diabetic animals, maltitol was able to significantly increase ($p<0.05$) digesta transit in the cecum only, while acarbose treatment significantly increased ($p<0.05$) digesta transit in the cecum and proximal colon (**Table 3.3**).

Table 3.3: Effect of maltitol ondigesta transit in the different segments of the intestinal tract during 1 h experimental period

Group	1[st]qtr	2[nd]qtr	3[rd]qtr	4[th]qtr	Cecum	Prox. Colon
			Small intestine			**Large Intestine**
			Digesta Transit (%)			
NC	95.26 ± 4.01	89.36 ± 4.58	70.27 ± 12.07[a]	48.53 ± 4.10[a]	23.68 ± 5.63[a]	36.81 ± 9.21[a]
NML	94.74 ± 4.15	86.75 ± 4.53	76.13 ± 3.34[b*]	73.23 ±3.53[b*]	35.35± 2.91[b*]	57.58 ±7.77[b*]
DBC	90.97 ± 0.08	87.86 ± 1.59	76.55 ± 5.16[b]	72.01 ±7.04[b]	37.56 ± 3.58[b]	49.69 ± 0.90[b]
DML	89.91 ± 7.57	84.27 ± 7.34	73.77 ± 2.86[b]	73.90±3.36[b]	52.14 ± 5.69[c#]	54.54 ± 2.61[b]
DBA	93.85 ± 4.72	87.01 ± 5.29	79.48 ± 6.09[b]	71.76 ±5.38[b]	65.26 ± 5.25[d#]	87.63 ± 3.07[c#]

Data are presented as mean ± SD of five to six animals. [abcd]Different superscript letters presented in each column for a given segment are significantly different from each other group of animals, *significantly different from NC group and #significantly different from DBC group ($p<0.05$; Tukey's HSD post-hoc test, IBM, SPSS version 21). NC: normal control; NML: normal maltitol; DBC: diabetic control; DML: diabetic maltitol; DBA: diabetic acarbose.

3.5   Discussion

Dietary sugar alcohols are known for their several health benefits in both normoglycemic and diabetic individuals (Livesey, 2003; Livesey 2012; Van Loveren, 2004). Maltitol is one of the few disaccharide sugar alcohols that are commonly used in various food products. However, the

effect of maltitol on intestinal glucose absorption and muscle glucose uptake has not been fully elucidated. In this study, we found that maltitol inhibits intestinal glucose absorption and increases insulin-mediated muscle glucose uptake under *ex vivo* condition but not in normal and type 2 diabetic rats, when co-ingested with glucose.

Dietary glucose is rapidly absorbed from the small intestine. However, *in vitro* studies have suggested that the proximal (Rider *et al*., 1967) or mid-small intestine (part of the duodenum and jejunum) (Lavin, 1976) exhibited the highest glucose absorption. Hence, the jejunal segment of rat intestine was used to study glucose absorption in the *ex vivo* experiment. Also, it is a known fact that osmotic pressure can influence intestinal water and glucose absorption. A previous *in vitro* study suggested that decreasing osmolality resulted in enhanced absorption of luminal water and glucose in isolated rat duodenum (Daum *et al*., 1978). In our *ex vivo* absorption study, the peak value of 5.20 ± 0.72 mg/cm jejunum of glucose absorption was observed which was reduced to 3.00 ± 0.35 mg/cm jejunum in the presence of 2.5% maltitol (**Fig. 3.1**). Additionally, the inhibitory effect of maltitol on the jejunal glucose absorption was concentration-dependent reaching the lowest value of 1.20 ± 0.20 mg/cm jejunum at 20% maltitol concentration. These findings suggest a concentration-dependent inhibitory effect of maltitol on jejunal glucose absorption under *ex vivo* condition, which may be partly due to the increasing osmolarity (388.48–896.68 mOsm/l) effect of increasing maltitol concentrations (2.5–10%).

However, unlike the significant *ex vivo* inhibitory effect of maltitol on jejunal glucose absorption, a single oral administration of maltitol, co-ingested with glucose, did not significantly affect or reduce the glucose absorption index in the intestinal segments (**Fig. 3.4**). This effect equally translates into the observed insignificant effect on postprandial blood glucose level (**Fig. 3.5**) of normoglycemic and diabetic animals. This observation under *in vivo* condition may be linked to the hydrolysis of maltitol by disaccharidases of the small intestinal mucosa as demonstrated in several previous studies (Kamoi, 1975; Rennhard & Bianchine, 1975; Lian-Loh *et al*., 1982). Interestingly, our *ex vivo* and *in vivo* observations seem to further explain the apparent discrepancy on the reported effects of maltitol on alpha glucosidase and alpha amylase which varied from *in vitro* (Kang *et al*., 2014) to *in vivo* experimental conditions (Matsuo, 2003).

Gastric emptying and digesta transit rates of GIT largely influence the absorption of nutrient in the small intestine. Previous studies have reported that delayed gastric emptying and accelerated digesta transit can contribute to reduce intestinal nutrient absorption as well as food intake (Salminen *et al*., 1984; Shafer *et al*., 1987), which is believed to be a mode of action of acarbose in controlling postprandial blood glucose rise in diabetic patients (Ranganath *et al*., 1998). In our *in vivo* absorption *study*, single oral dose of maltitol did not significantly influence gastric emptying in normal or diabetic animals (**Fig. 3.3**), which may be partly responsible for the insignificant effect of maltitol on small intestinal glucose absorption (**Fig. 3.4**). However, maltitol accelerated the digesta transit in the cecum of diabetic rats but not in other segments (**Table 3.3**), which could not affect the overall intestinal glucose absorption since most of the glucose absorption occur in the 1st qtr to mid-small intestine (Rider *et al*., 1967; Lavin, 1976).

The uptake of circulating glucose by cells, for either storage or energy metabolism is a major mechanism of the body to maintain glucose homeostasis when there is rise in blood glucose due to dietary glucose absorption, glycogen breakdown and gluconeogenesis (Aronoff *et al*., 2004). Insulin is the major regulatory hormone that stimulates clearance of circulating glucose via an insulin-mediated cellular uptake of glucose (Aronoff *et al*., 2004). Although in some previous studies it has been reported that hyperosmolarity increases muscle glucose uptake via modulating AMP-Kinase and/or inhibiting the endocytosis of glucose transporter type 4 (Gual *et al*., 2003), this may not be the case in our *ex vivo* study. The data from our *ex vivo* study (**Table 3.1** and **Fig. 3.2**) revealed that although maltitol exhibited insulin-mediated glucose uptake (GU_{50} = 7.31 ± 2.08%), it did not show any appreciable glucose uptake effect without insulin in isolated rat psoas muscle (GU_{50} = 111.12 ± 19.36%). This observation could suggest, at least in *ex vivo* condition, that maltitol may potentiate an insulin-mediated glucose uptake in muscles, when increasing osmolarity (388.48–896.68 mOsm/l) due to increasing maltitol concentrations may not be an influencing factor in this regard.

In summary, maltitol showed significant inhibitory effects on small intestinal glucose absorption and could increase insulin-mediated muscle glucose uptake *ex vivo* but did not possess appreciable inhibitory effect on intestinal glucose absorption or postprandial blood glucose level in normal or diabetic animals, when co-ingested with glucose, which may be linked to its hydrolysis by intestinal disaccharidases. Thus, maltitol may not be a useful dietary

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supplement to ameliorate hyperglycemia via reducing intestinal glucose absorption or enhancing muscle glucose uptake in normal or diabetic condition.

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CHAPTER 4

MYO-INOSITOL INHIBITS INTESTINAL GLUCOSE ABSORPTION AND PROMOTES MUSCLE GLUCOSE UPTAKE: A DUAL APPROACH STUDY

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3.1   Abstract

The present study investigated the effects of myo-inositol on muscle glucose uptake and intestinal glucose absorption *ex vivo* as well as in normal and type 2 diabetes model of rats. In the *ex vivo* study, both intestinal glucose absorption and muscle glucose uptake were studied in isolated rat jejunum and psoas muscle respectively in the presence of increasing concentrations (2.5 % to 20 %) of myo-inositol. In the *in vivo* study, the effect of a single bolus dose (1 g/kg bw) of oral myo-inositol on intestinal glucose absorption, blood glucose, gastric emptying and digesta transit was investigated in normal and type 2 diabetic rats after 1 h of co-administration with 2 g/kg bw glucose, when phenol red was used as a recovery marker. Myo-inositol inhibited intestinal glucose absorption ($IC_{50} = 28.23 \pm 6.01$ %) and increased muscle glucose uptake, with ($GU_{50} = 2.68 \pm 0.75$ %) or without ($GU_{50} = 8.61 \pm 0.55$ %) insulin. Additionally, oral myo-inositol not only inhibited duodenal glucose absorption and reduced blood glucose increase, but also delayed gastric emptying and accelerated digesta transit in both normal and diabetic animals. Results of this study suggest that dietary myo-inositol inhibits intestinal glucose absorption both in *ex vivo* and in normal or diabetic rats and also promotes muscle glucose uptake *in ex vivo* condition. Hence, myo-inositol may be further investigated as a possible anti-hyperglycemic dietary supplement for diabetic foods and food products.

4.2   Introduction

Recent data indicate that about 415 million people have diabetes worldwide and this figure is likely to increase to about 642 million by 2040 (International Diabetes Federation, 2015). Among the two major types of diabetes (type 1 and type 2), type 2 is the most prevalent one, accounting for more than 90 % of all diabetic cases (Loghmani, 2005). Type 2 diabetes (T2D) is a heterogeneous disorder characterized by insulin resistance followed by partial pancreatic β-cell dysfunction (Partley, 2013), leading to persistent hyperglycaemia. The chronic hyperglycaemia is strongly influenced by sedentary life style, high fat and high calorie diets as well as routine consumption of refined and simple sugars (Uusitupa, 2002; Hu, 2003; Montonen *et al*., 2007; Stanhope *et al*., 2011). Hence, among other therapeutic approaches, reducing the consumption of high calorie and high glycaemic index foods as well as increasing intake of agents that delay

post-prandial hyperglycaemia are rapidly gaining public endorsement (Bessesen, 2001; Livesey, 2003; Brown *et al*., 2010). Recently, there has been a growing interest in the search for nutraceuticals such as sugar alcohols that possess lower calorific value and glycaemic index compared to sucrose and could have the potentials to delay the postprandial blood glucose rise (Livesey, 2003; Brown *et al*., 2010).

Myo-inositol is one of the rarely used sugar alcohols, which has structural resemblance to glucose and it is present in a wide variety of foods and food products at low concentrations (Clements & Darnell, 1980). It belongs to the group of stereoisomers collectively called inositol (1,2,3,4,5,6-cyclohexanehexol or simply cyclohexanehexol) (Chung & Kwon, 1999). Inositol comprises of nine different stereoisomers but myo-inositol is the body's main stereoisomer (comprising over 90 % of cellular inositol) (Chung & Kwon, 1999; McLaurin *et al*., 2000). However, D-chiro-inositol has been reported as another important stereoisomer of myo-inositol in fat, muscle and liver tissues (Facchinetti *et al*., 2015). The action of inositol epimerase determines the physiological distribution of myoinositol and D-chiro-inositol, which also influences their distinct physiological functions (Heimark *et al*., 2014; Unfer *et al*., 2014; Facchinetti *et al*., 2015).

Myo-inositol is associated with glucose transporters activation and glucose utilization, while D-chiro-inositol is majorly involved in the synthesis of glycogen in the liver, fat and muscle tissues (Heimark *et al*., 2014). A number of recent studies reported that these two inositols consistently improved glucose metabolic markers, insulin resistance and dyslipidaemia in post postmenopausal women with metabolic syndrome (Giordano *et al*., 2011; Nordio & Proietti, 2012; Minozzi *et al*., 2013), when their combination normalized the risks of metabolic diseases better than myo-inositol alone in overweight patients with polycystic ovary syndrome (PCOS) (Nordio & Proietti, 2012; Minozzi *et al*., 2013). From the results of a recent international consensus conference, myo-inositol and D-chiroinositol have been reported as the second messenger of insulin in several insulin dependent processes such as metabolic syndrome and PCOS (Facchinetti *et al*., 2015). It has been also reported that although these two isomers of inositol have distinct effects, a number of observations required further intensive investigation.

Additionally, myo-inositol supplementation has been reported to modulate lipid and glucose metabolism, which are closely associated with T2D (Onomi & Katayama, 1997; Yap *et*

al., 2007; Maeba *et al.*, 2008; Dang *et al.*, 2010). Daily administration of 5 or 10 g/ day of myo-inositol for a week significantly reduced LDL-cholesterols and apo-lipoprotein B in subjects with lifestyle-related diseases such as obesity and hyperlipidemia (Maeba *et al.*, 2008). In the same study, significant reduction of blood glucose level, particularly in the metabolic syndrome subjects, suggests the hypoglycaemic potential of myo-inositol. Additionally, Dang *et al.* (2010) reported that oral administration of a single dose of 1000 mg/kg myo-inositol caused an acute hypoglycaemic effect when administered with 2 g/kg bw glucose, and was also responsible for Glut-4 translocation, which was observed 30 min after the ingestion time (Dang *et al.*, 2010). In fact, 1 mM of myo-inositol and/or several inositol metabolites have been reported to be as effective as 100 nM insulin in stimulating Glut-4 translocation to the cell membrane, *in vitro* (Yap *et al.*, 2007), which may be partly associated with the improved insulin sensitivity and hypoglycemic effects of dietary myo-inositol.

In spite of the above-mentioned studies, the direct effect of dietary myo-inositol on muscle glucose uptake is still not clear, and there are currently no studies to ascertain the effect of dietary myo-inositol on intestinal glucose absorption. Hence, the present study was conducted to examine the effects of myo-inositol on muscle glucose uptake and intestinal glucose absorption in isolated rat's psoas muscle and jejunum, respectively (*ex vivo*) and in normal and type 2 diabetes model of rats (*in vivo*).

4.3   Materials and methods

Please refer to chapter 2, sub-sections 2.2.1 and 2.2.2, pages 66-74 for detailed material and methods of this article.

4.4 Results

4.4.1 *Ex vivo* study (experiment 1)

Effects of myo-inositol on intestinal glucose absorption and muscle glucose uptake

Data for the effects of myo-inositol on intestinal glucose absorption and muscle glucose uptake are presented in **Fig. 4.1** and **4.2** as well as **Table 4.1**. Myo-inositol exhibited a concentration-dependent effect on rat jejunal glucose absorption *ex vivo* (**Fig. 4.1**) with an IC50 = 28.30 ± 6.01 %. Incubation of jejunal tissue in 10 % and 20 % myo-inositol significantly ($p < 0.05$) reduced glucose absorption compared to the control. However, the effects of 2.5 % and 5 % myo-inositol on the jejunal glucose absorption were not significantly ($p > 0.05$) different compared to the control (**Fig. 4.1**).
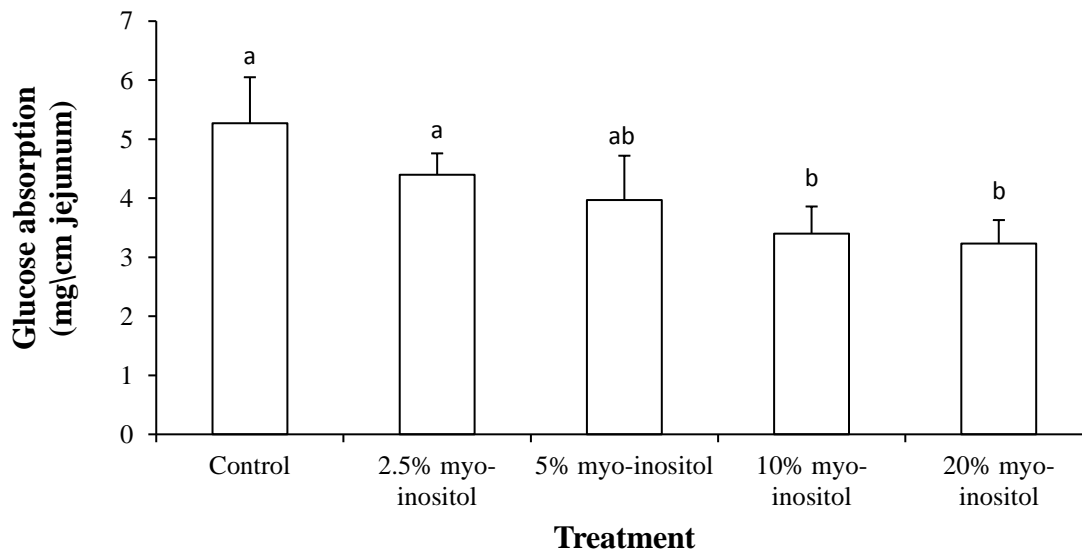
Figure 4.1: Effect of myo-inositol on glucose absorption in isolated rat jejunum. Data are presented as mean $\pm$ SD of five replicates of analysis. [ab]Different letters presented above the bars are significantly different from each other ($p < 0.05$; Tukey's HSD multiple range post-hoc test, IBM, SPSS version 21).

Furthermore, glucose uptake at 10 % and 20 % concentrations of myo-inositol was significantly higher ($p < 0.05$) than the control, when glucose uptake at 2.5 % and 5 % of myo-inositol containing sample did not show any significant difference compared to the control with or without insulin (**Fig. 4.2**). A 2 mg/mL metformin significantly increased ($p < 0.05$) muscle glucose uptake than all myo-inositol containing samples with or without insulin. The computed GU50 values for the increased glucose uptake in the isolated rat psoas muscle by myo-inositol (2.5 % - 20 %) were 2.68 ± 0.75 %, with insulin and 8.61 ± 0.55 %, without insulin (**Table 4.1**), which showed that insulin significantly increased the muscle glucose uptake activity of myo-inositol.

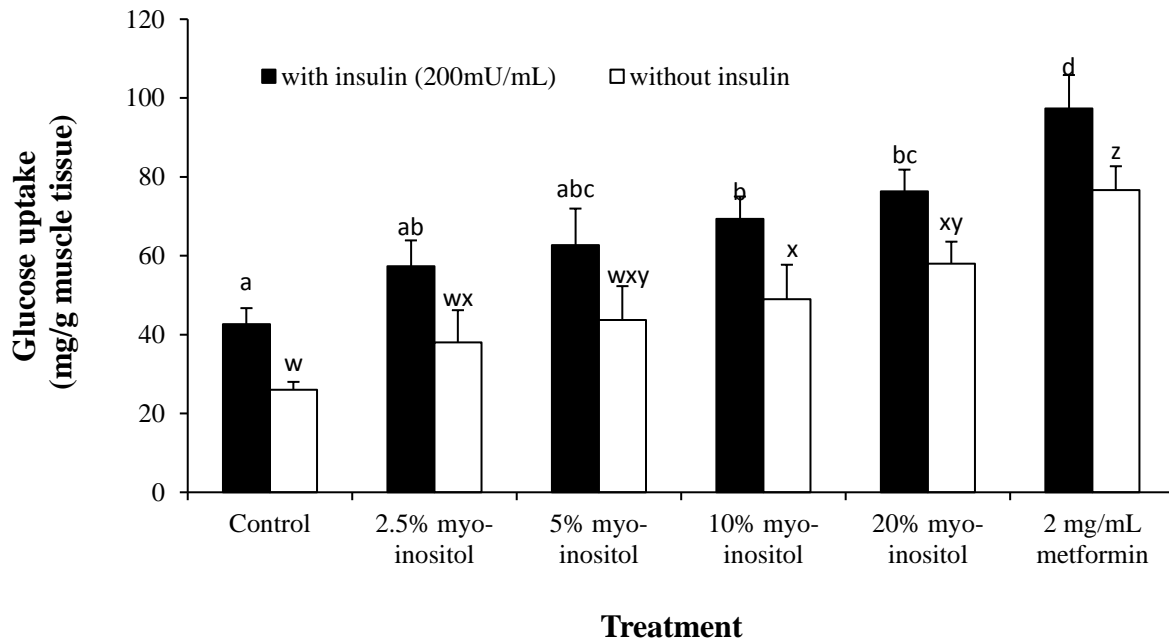**Figure 4.2:** Effect of myo-inositol on glucose uptake with or without insulin in isolated rat psoas muscle. Data are presented as mean ±SD of triplicate analysis. [a-d or w-z] Different letters presented above the bars for with or without insulin are significantly different from each other ($p < 0.05$; Tukey's HSD multiple range post-hoc test, IBM, SPSS version 21).

Table 4.1: Percentage GU_{50} values of glucose uptake increase of myo-inositol in isolated psoas muscle, with and without insulin

	With insulin (200mU/mL)	Without insulin
GU_{50} (%)*	2.68 ± 0.75^a	8.61 ± 0.55^b

Data are presented as mean $\pm$ SD of triplicate analysis. [ab]Values with different superscript letters are significantly different from each other ($p < 0.05$; Tukey's HSD multiple range *post-hoc* test, IBM, SPSS version 21).*GU_{50}: Concentration (% as w/v) of myo-inositol required to cause 50% increase of muscle glucose uptake.

Additionally, muscle tissue viability studies confirmed that 1 h incubation of psoas muscle tissue did not significantly affect the viability of the tissue (Please refer to **Table 3.2** in chapter 3, sub-section 3.4.1, section 3.4, page 92 for supporting data).

4.4.2   *In vivo* intestinal glucoseabsorption study (experiment 2)

Effects of myo-inositol on gastric emptying, glucose absorption index, digesta transit and blood glucose level

The data for the effects of myo-inositol on gastric emptying, glucose absorption index and blood glucose level are presented in **Fig. 4.3** to **4.5** and **Table 4.2**. Gastric emptying significantly increased ($p < 0.05$) after the induction of diabetes, while myo-inositol and acarbose treatments significantly reduced ($p < 0.05$) gastric emptying in diabetic animals. Treatment with myo-inositol did not significantly affect gastric emptying in normal animals (**Fig. 4.3**).

Furthermore, Ingestion of myo-inositol significantly reduced ($p < 0.05$) GAI in the 1st qtr of the small intestine in both normal and diabetic animals, while similar effects were observed in the 2nd and 3rd qtrs of the small intestine of normal animals. Ingestion of acarbose significantly reduced ($p < 0.05$) GAI in the 1st qtr of the small intestine, cecum as well as proximal colon of diabetic animals, while no significant difference in GAI was observed in the other intestinal segments of the different animal groups (**Fig. 4.4**).
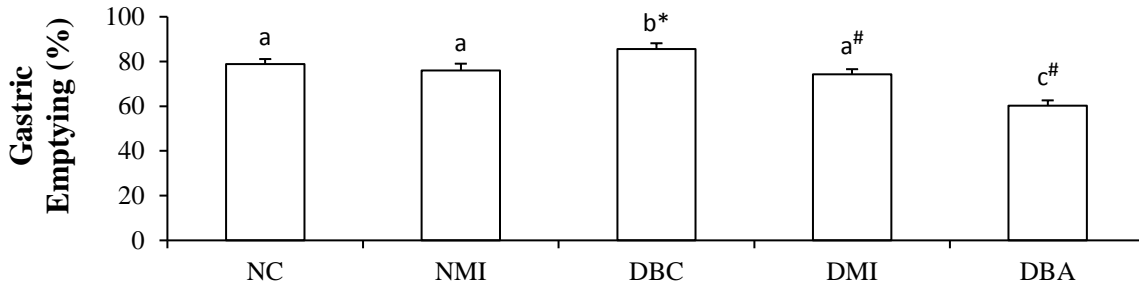
Figure 4.3: Effect of myo-inositol on gastric emptying in different animal groups at the end of a 1 h experimental period. Data are presented as mean ± SD of 5–6 animals. [abc]Different letters presented above the bars are significantly different from each other group of animals, *significantly different from NC group and #significantly different from DBC group (p<0.05; Tukey's HSD post-hoc test, IBM, SPSS version 21). NC, normal control; NMI, normal myo-inositol; DBC, diabetic control; DMI, diabetic myo-inositol; DBA, diabetic acarbose.
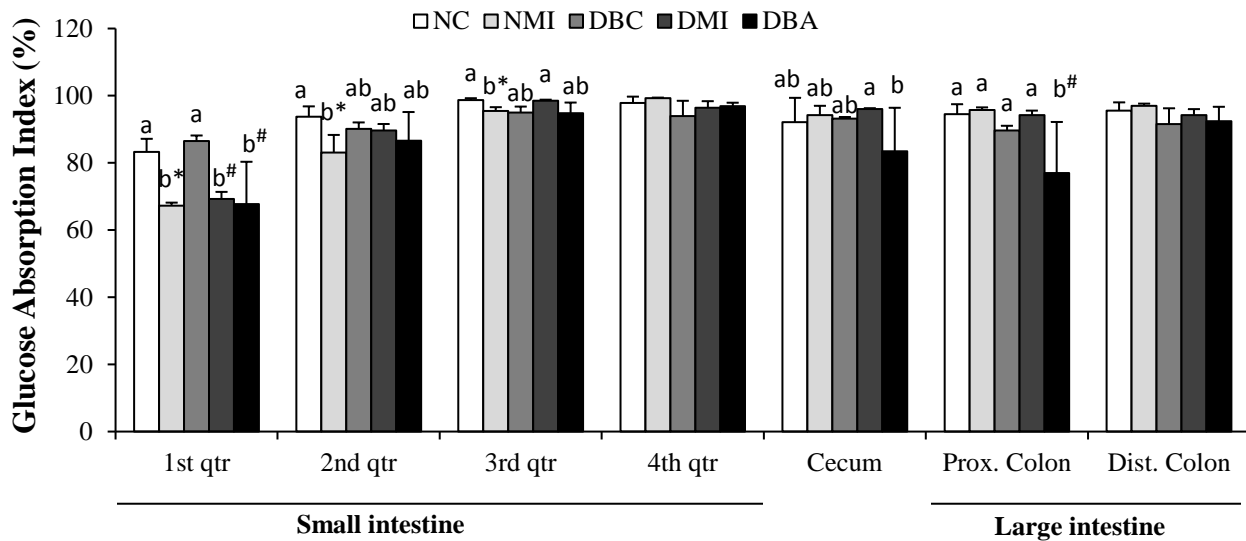
Figure 4.4: Effect of myo-inositol on glucose absorption index (GAI) in the different GIT segments of different animal groups at the end of a 1 h experimental period. Data are presented as mean ± SD of five to six animals. [ab]Different letters presented above the bars for a given segment are significantly different from each other group of animals, *significantly different from NC group and #significantly different from DBC group (p<0.05; Tukey's HSD post-hoc test, IBM, SPSS version 21). NC, normal control; NMI, normal myo-inositol; DBC, diabetic control; DMI, diabetic myo-inositol; DBA, diabetic acarbose.

Although the data were not significantly different in normal animals, single oral dose of myo-inositol and acarbose significantly reduced (p < 0.05) the increment of blood glucose 1 h after the dose administration (**Fig. 4.5**).
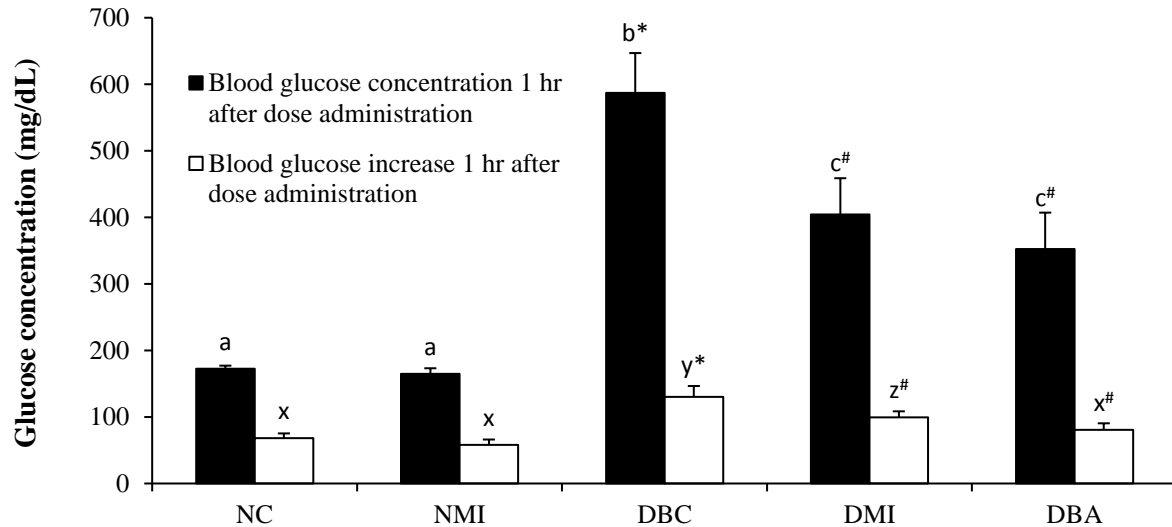
Figure 4.5: Effects of myo-inositol on final blood glucose concentrations and blood glucose increase in animals after 1 h of the dose administration. Data are presented as mean ± SD of six animals. abc& xyzDifferent letters presented above the bars are significantly different from each other group of animals,*significantly different from NC group and #significantly different from DBC group (p<0.05; Tukey's HSD multiple range post-hoc test, IBM, SPSS version 21). NC, normal control; NMI, normal myo-inositol; DBC, diabetic control; DMI, diabetic myo-inositol; DBA, diabetic acarbose.

Although not significantly different (p > 0.05), myoinositol treatment increased digesta transit in the 1st, 3rd and 4th qtrs of the small intestine of diabetic animals as well as in the 2nd qtr of the small intestine of normal animals (**Table 4.2**). However, both myo-inositol and acarbose treatments significantly increased (p < 0.05) digesta transit in the cecum and proximal colon of the diabetic animals. On the other hand, myoinositol treatment significantly increased (p < 0.05) digesta transit from the 3rd qtr of small intestine to proximal colon of normal animals.

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Table 4.2: Effect of myo-inositol on digesta transit in the different segments of the intestinal tract during 1 h experimental period

Group	1stqtr	2ndqtr	3rdqtr	4thqtr	Cecum	Prox. colon
	Small intestine					Large intestine
	Digesta transit (%)					
NC	95.26 ± 4.01^{ab}	89.36 ± 4.58	70.27 ± 12.07^{a}	48.53 ± 4.10^{a}	23.68 ± 5.63^{a}	36.81 ± 9.21^{a}
NMI	95.52 ± 1.13^{a}	92.97 ± 2.37	$88.84 \pm 1.71^{b*}$	$80.92 \pm 3.53^{b*}$	$78.13 \pm 7.57^{b*}$	$68.42 \pm 6.73^{b*}$
DBC	90.97 ± 0.08^{b}	87.86 ± 1.59	76.55 ± 5.16^{ab}	72.01 ± 7.04^{b}	37.56 ± 3.58^{c}	49.69 ± 0.90^{c}
DMI	95.22 ± 2.72^{ab}	87.40 ± 5.89	85.67 ± 1.16^{ab}	82.88 ± 3.94^{b}	$51.98 \pm 1.34^{d\#}$	$55.03 \pm 3.43^{b\#}$
DBA	93.85 ± 4.72^{ab}	87.01 ± 5.29	79.48 ± 6.09^{ab}	71.76 ± 5.38^{b}	$65.26 \pm 5.25^{b\#}$	$87.63 \pm 3.07^{d\#}$

Data are presented as mean $\pm$ SD of five to six animals. ^{abcd}Different superscript letters presented in each column for a given segment are significantly different from each other group of animals, *significantly different from NC group and #significantly different from DBC group ($p<0.05$; Tukey's HSD post-hoc test, IBM, SPSS version 21). NC, normal control; NMI, normal myo-inositol; DBC, diabetic control; DMI, diabetic myo-inositol; DBA, diabetic acarbose.

4.5   Discussion

Myo-inositol is a sugar alcohol that is used as a supplement in various foods and food products. Studies have suggested that myo-inositol may be effective in ameliorating diabetes, obesity and metabolic syndrome related abnormalities via improving insulin action as well as glucose and lipid metabolism (Onomi & Katayama, 1997; Yap *et al*., 2007; Maeba *et al*., 2008; Dang *et al*., 2010; Giordano *et al*., 2011; Facchinetti *et al*., 2015). In the present study, it was observed that myo-inositol inhibited intestinal glucose absorption in isolated rat jejunum and in the proximal small intestine of normal and diabetic rats, while it dose-dependently increased glucose uptake in isolated rats psoas muscle (**Figs. 4.1, 4.2** & **4.4**), which may be possible mechanisms of its anti-hyperglycemic potential.

In glucose metabolism, insulin enhances the clearance of circulating glucose for either storage or energy production in cells, which ensures blood glucose homeostasis (Aronoff *et al*., 2004). Hence, in type 2 diabetes, insulin resistance or insensitivity is the major reason why

actively respiring cell like muscle cells do not respond to insulin signaling to take up glucose, causing impaired glucose tolerance and persistent hyperglycaemia (Dedoussis *et al*., 2007). Myoinositol is known to be involved in glucose metabolism, especially in insulin-mediated glucose uptake via its metabolites such as P-type inositol phosphoglycans (Saltiel, 1990; Kunjara *et al*., 1999; McLean *et al*., 2008; Facchinetti *et al*., 2015). Accordingly, dietary myo-inositol has been reported to be associated with the improvement of biomarkers of insulin sensitivity in individuals with diabetes related abnormalities (Maeba *et al*., 2008; Giordano *et al*., 2011) and translocation of Glut-4 to cell membranes *in vitro* (Yap *et al*., 2007) and *in vivo* (Dang *et al*., 2010).

Since improved insulin sensitivity mainly translates into the modulation of cellular glucose uptake, we investigated the effect of myo-inositol on glucose uptake in isolated rat psoas muscle (with or without insulin) as an index to further ascertain the ability of dietary myoinositol to improve insulin sensitivity in diabetic condition. Interestingly, our data showed that myo-inositol (with or without insulin) markedly increased muscle glucose uptake at *ex vivo* condition in a concentration-dependent manner relative to the control (**Fig. 4.2** and **Table 4.1**). This observation could suggest, at least in *ex vivo* condition, that myo-inositol may potentiate an insulin-mediated glucose uptake in muscles. However, it is also worthy to note that the interpretation of this observation or effect of myo-inositol is only limited to an *ex vivo* context and does not directly translate into an *in vivo* glucose uptake effect, but further supports the potential usefulness of myo-inositol supplementation in improvement of insulin sensitivity in the skeletal muscles (Dang *et al*., 2010; Facchinetti *et al*., 2015). Thus, further in depth studies are needed to adequately elucidate and confirm this *ex vivo* muscle glucose uptake potential of myo-inositol at *in vivo* condition.

On the other hand, the absorption of dietary glucose varies across the different segments of the small intestine. It was previously suggested that the proximal to mid-small intestine (part of the duodenum and jejunum) is responsible for the absorption of the majority of dietary glucose (Rider *et al*., 1967; Riesenfeld *et al*., 1980); hence, tissue from this segment was used to study glucose absorption in the *ex vivo* part of this study. According to the results from our *ex vivo* study, myo-inositol caused a concentration-dependent decrease in jejunal glucose absorption (**Fig. 4.1**) which suggests the potential of myo-inositol to impede postprandial blood glucose rise.

Interestingly, this was further confirmed in the *in vivo* section of our study where myo-inositol significantly reduced ($p < 0.05$) glucose absorption index (GAI) in the 1st qtr of the small intestine of normal and diabetic rats (**Fig. 4.4**), which may have translated into the lower blood glucose increase observed in myo-inositol fed rats, particularly in the diabetic group (**Fig. 4.5**). Indeed, the observed potent action of the dietary myo-inositol in the 1st qtr of the small intestine of animals is noteworthy because it is the site with relatively high glucose absorption capacity (Rider *et al*., 1967). Thus, suggesting that myo-inositol may have the potentials to inhibit the absorption of an appreciable portion of glucose obtained from the breakdown of starch and other complex carbohydrates.

Additionally, gastric emptying and digesta transit rates of GIT content largely influence the absorption of nutrient from the small intestine. Previous studies have reported that delayed gastric emptying and accelerated digesta transit can contribute to reduce intestinal nutrient absorption as well as food intake (Salminen *et al*., 1984; Shafer *et al*., 1987), which has been hypothesized to be a mode of action of acarbose in controlling postprandial blood glucose rise in diabetic patients (Ranganath *et al*., 1998). Data from our present study showed that gastric emptying rate was significantly increased in animals after the induction of diabetes (**Fig. 4.3**), which depicts the clinical presentation of gastric emptying in most type 2 diabetic states (Phillips *et al*., 1991; Horowitz *et al*., 1996). Our study also revealed that dietary myo-inositol significantly delayed the diabetes-induced elevation of gastric emptying time in rats (**Fig. 4.3**) and concomitantly increased small intestinal and colonic digesta transit rates in normal and diabetic rats (**Table 4.2**), both of which might largely contribute to the reduced intestinal glucose absorption in normal or diabetic animals treated with myo-inositol. Based on the above observations, it is possible to hypothesize that myo-inositol may perhaps act via a competitive inhibition mechanism as a result of its mild structural resemblance with glucose. However, further study is needed to confirm this hypothesis.

In summary, results from our study showed that myoinositol inhibited jejunal glucose absorption and increased muscle glucose uptake in an *ex vivo* condition. Our *in vivo* data demonstrated the inhibitory effect of orally administered myo-inositol on proximal small intestinal glucose absorption, which may be partly due to the observed delayed gastric emptying and accelerated digesta transit. Although our *in vivo* data also showed lower postprandial blood

glucose increase in myo-inositol fed animals, particularly in the diabetic group, it cannot be confirmed from our study whether the inhibition of intestinal glucose absorption was solely responsible for this effect or not. It could be hypothesized that myoinositol also influenced postprandial blood glucose increase by exerting muscle glucose uptake effect as seen in our *ex vivo* study. However, this hypothesis can only be confirmed following further in depth studies, since our muscle glucose uptake study was limited to an *ex vivo* state only.

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CHAPTER 5

SORBITOL INCREASES MUSCLE GLUCOSE UPTAKE *EX VIVO* AND INHIBITS INTESTINAL GLUCOSE ABSORPTION *EX VIVO* AND IN NORMAL AND TYPE 2 DIABETIC RATS

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Preface: The Effects of sorbitol on intestinal glucose absorption and muscle glucose uptake was investigated in this article using *ex vivo* and in *vivo* experimental models. This article has been published in to Applied Physiology, Nutrition and Metabolism, 2016. doi: 10.1139/apnm-2016-0433).

5.1 Abstract

Previous studies have suggested that sorbitol, a known polyol sweetener possesses glycemic control potentials. However, the effect of sorbitol on intestinal glucose absorption and muscle glucose uptake still remains elusive. The present study investigated the effects of sorbitol on intestinal glucose absorption and muscle glucose uptake as possible anti-hyperglycemic or glycemic control potentials using *ex vivo* and *in vivo* experimental models. Sorbitol (2.5% to 20%) inhibited glucose absorption in isolated rat jejuna ($IC_{50} = 14.6 \pm 4.6\%$) and increased glucose uptake in isolated rat psoas muscle with ($GU_{50} = 3.5 \pm 1.6\%$) or without insulin ($GU_{50} = 7.0 \pm 0.5\%$) in a concentration-dependent manner. Furthermore, sorbitol significantly delayed gastric emptying, accelerated digesta transit, inhibited intestinal glucose absorption and reduced blood glucose increase in both normoglycemic and type 2 diabetic rats after 1 h of co-ingestion with glucose. Data of this study suggest that sorbitol exhibited anti-hyperglycemic potentials, possibly via increasing muscle glucose uptake *ex vivo*and reducing intestinal glucose absorption in normal and type 2 diabetic rats. Hence, sorbitol may be further investigated as a possible anti-hyperglycemic sweetener.

5.2 Introduction

Diabetes is one of the major global public health problems and its prevalence is rapidly increasing in all over the world, particularly in the developing nations. According to the International Diabetes Federation, about 415 million people have diabetes worldwide and this figure is likely to become 642 million by 2040 (International Diabetes Federation, 2015). Among two major types of diabetes, type 2 diabetes (T2D) is the most prevalent one, which accounts for more than 90% of all diabetic cases (Loghmani, 2005). T2D is a heterogeneous disorder characterized by insulin resistance followed by partial pancreatic beta-cell dysfunction (Partley, 2013). It is strongly influenced by sedentary life style, high fat and high calorie diet intake as well as routine consumption of refined and simple carbohydrates e.g. sucrose, fructose and so on (Uusitupa, 2002; Hu, 2003; Montonen *et al*., 2007; Stanhope *et al*., 2011). Excessive dietary intake of sucrose- and fructose-containing foods and food products has been consistently associated with detrimental health outcomes, such as elevated food intake, weight gain,visceral

adiposity, reduced insulin sensitivity, lipid dysregulation and the risk of developing T2D (Thorburn*et al*., 1989; Astrup *et al*., 2002; Elliott *et al*., 2002; Stanhope *et al*., 2011). Hence, there has been an upsurge on the use of alternative sweetening agents, such as sugar alcohols.

Sugar alcohol is one of the classes of sugar substitute that have gained great popularity, especially for overweight, obese and diabetic individuals due to their sweetening ability with lesser undesired calories compared to sucrose (Islam and Indrajit, 2012). Additionally, apart from their minimal effect on the integrated blood glucose level compared to sucrose (Talbot and Fisher, 1978; Livesey, 2003), most commonly used sugar alcohols have demonstrated oral health care usefulness (Mäkinen, 2010; Gupta *et al*., 2013). Sorbitol is one of the widely used sugar alcohols. Several studies have reported that sorbitol possesses glycemic control effects, but may also possess anti-hyperglycemic effects (Brunzell, 1978; Livesey, 2003). In a previous study, it has been reported that a 35 g of orally administered sorbitol caused significantly lower increment in plasma glucose compared to sucrose or fructose in both normal and diabetic human subjects (Akgün and Ertel, 1980). In a recent study, it has been reported that some commonly used sugar alcohols including sorbitol exhibited *in vitro* alphaglucosidase, alpha amylase and sucrase inhibitory activities (Kang *et al*., 2014). Additionally, after the evaluation of several studies, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in a final comment or remark reported that sorbitol may inhibit glucose absorption as well (WHO, 1987).

Although some previous and recent studies suggested the glycemic control or anti-hyperglycemic potentials of sorbitol *in vivo* and alpha glucosidase, alpha amylase and sucrase inhibitory activities *in vitro*, the effect of sorbitol on intestinal glucose absorption and muscle glucose uptake still remains unknown. Hence, the present study was conducted to examine the effects of sorbitol on intestinal glucose absorption and gastric emptying as well as muscle glucose uptake as possible anti-hyperglycemic mode of actions by using some *ex vivo* and *in vivo* experimental models.

5.3   Materials and methods

Please refer to chapter 2, sub-sections 2.2.1 and 2.2.2, pages 66-74 for detailed material and methods of this article.

5.4   Results

5.4.1   *Ex vivo* study (experiment 1)

Effects of sorbitol on intestinal glucose absorption and muscle glucose uptake

Data for the effects of sorbitol on intestinal glucose absorption and muscle glucose uptake are presented in **Fig. 5.1** and **5.2** as well as **Table 5.1**. The data showed a concentration-dependent effect of sorbitol on jejunal glucose absorption *ex vivo* (**Fig. 5.1**). Incubation of jejunal tissue in 5%, 10% and 20% sorbitol significantly reduced glucose absorption ($p<0.05$) compared to the control, when no significant difference was observed between the control and 2.5% sorbitol containing sample. Additionally, no significant difference was observed between the 5%, 10% and 20% sorbitol containing samples, while glucose absorption at 20% sorbitol concentration was significantly reduced ($p<0.05$) compared to glucose absorption at 2.5% sorbitol concentration (**Fig. 5.1**).
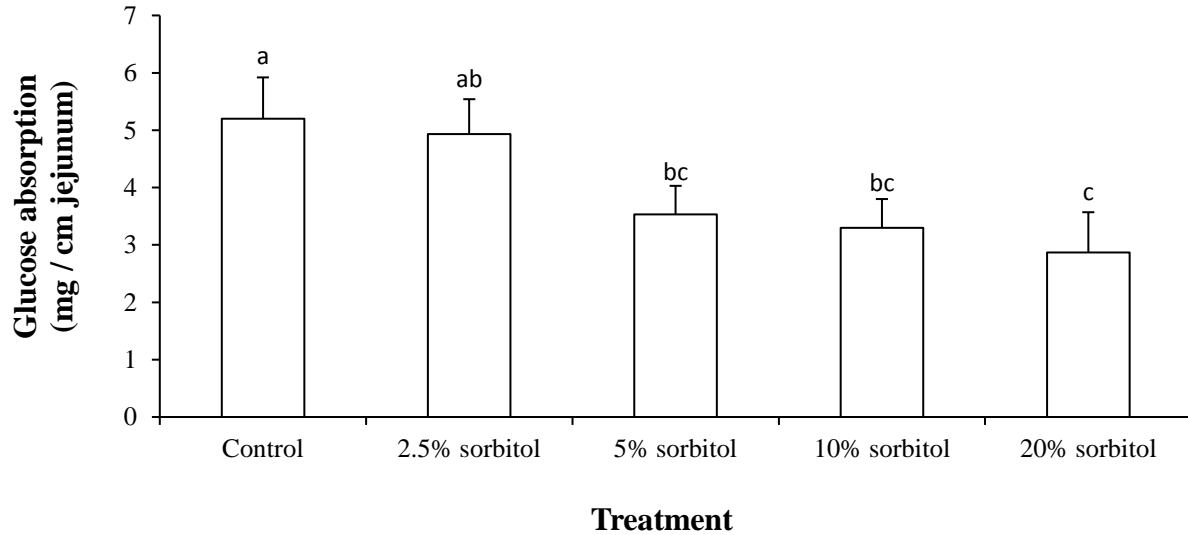

Figure 5.1: Effect of sorbitol on glucose absorption in isolated rat jejunum. Data are presented as mean ± SD of five replicates of analysis. [abc]Different letters presented above the bars are significantly different from each other ($p < 0.05$; Tukey's HSD multiple range post-hoc test, IBM, SPSS version 21).

Sorbitol (2.5% to 20%) increased dose-dependent glucose uptake in the isolated rat psoas muscle with ($GU_{50} = 3.53 \pm 1.61\%$) or without ($GU_{50} = 7.02 \pm 0.46\%$) insulin. Glucose uptake at 10% and 20% concentrations of sorbitol was significantly higher (p<0.05) than the control and 2.5% sorbitol containing sample. No significant difference in glucose uptake was observed between the 5% and 20% sorbitol containing samples with or without insulin (**Fig. 5.2**). A 2mg/mL metforminsignificantly increasedmuscle glucose uptake than all sorbitol containing samples with or without insulin, while incubation with insulin significantly influenced (p<0.05) the glucose uptake effect of sorbitol (**Table 5.1**). Similar effects of metformin were found in the previous studies as well (Turban *et al*., 2012; Kristensen *et al*., 2014).
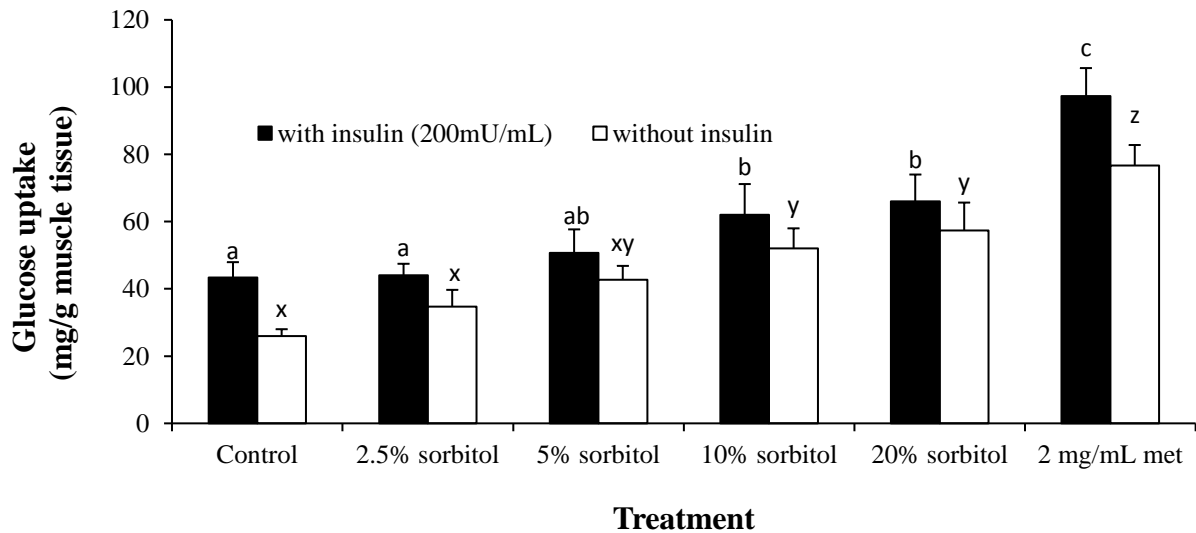

Figure 5.2: Effect of sorbitol on glucose uptake with or without insulin in isolated rat psoas muscle. Data are presented as mean ±SD of triplicate analysis. [a-c or x-z] Different letters presented above the bars for with or without insulin are significantly different from each other (p < 0.05; Tukey's HSD multiple range post-hoc test, IBM, SPSS version 21).

Additionally, muscle tissue viability studies confirmed that 1 h incubation of psoas muscle tissue did not significantly affect the viability of the tissue (Please refer to **Table 3.2** in chapter 3, sub-section 3.4.1, section 3.4, page 92 for supporting data).

Table 5.1: Percentage GU_{50} values of glucose uptake increase of sorbitol in isolated psoas muscle, with and without insulin

	With insulin [200mU/mL]	Without insulin
GU_{50} [%]*	3.53 ± 1.61^a	7.02 ± 0.46^b

Data are presented as mean $\pm$ SD of triplicate analysis. ^{ab}Values with different superscript letters are significantly different from each other (p < 0.05; Tukey's HSD multiple range *post-hoc*test, IBM, SPSS version 21). *GU_{50}: Concentration (% as w/v) of sorbitol required to cause 50% increase of muscle glucose uptake.

5.4.2 *In vivo* intestinal glucoseabsorption study (experiment 2)

Effects of sorbitol on gastric emptying, glucose absorption index, digesta transit and blood glucose level

The data for the effects of myo-inositol on gastric emptying, glucose absorption index and blood glucose level are presented in **Fig. 5.3** to **5.5** and **Table 5.2**. Gastric emptying significantly increased (p<0.05) after the induction of diabetes but significantly decreased (p<0.05) after the treatment with either sorbitol or acarbose (**Fig. 5.3**). Significantly slower gastric emptying of diabetic animals was observed in the acarbose ingested group compared to the sorbitol ingested groups. Treatment with sorbitol did not significantly affect gastric emptying in normal animals (Fig. 5.3).

Furthermore, administration of either sorbitol or acarbose significantly influenced GAI in the different intestinal segments of both normal and diabetic animals (**Fig. 5.4**). Ingestion of sorbitol significantly reduced (p<0.05) GAI in the 1st and 2nd qtrs of the small intestine in both normal and diabetic animals, while ingestion of acarbose significantly reduced (p<0.05) GAI in the 1stqtr of the small intestine of diabetic animals. In the proximal colon sorbitol and acarbose treatment significantly reduced (p<0.05) GAI in normal and diabetic animals respectively, while no significant difference was observed in the other intestinal segments of different animal groups (**Fig. 5.4**).
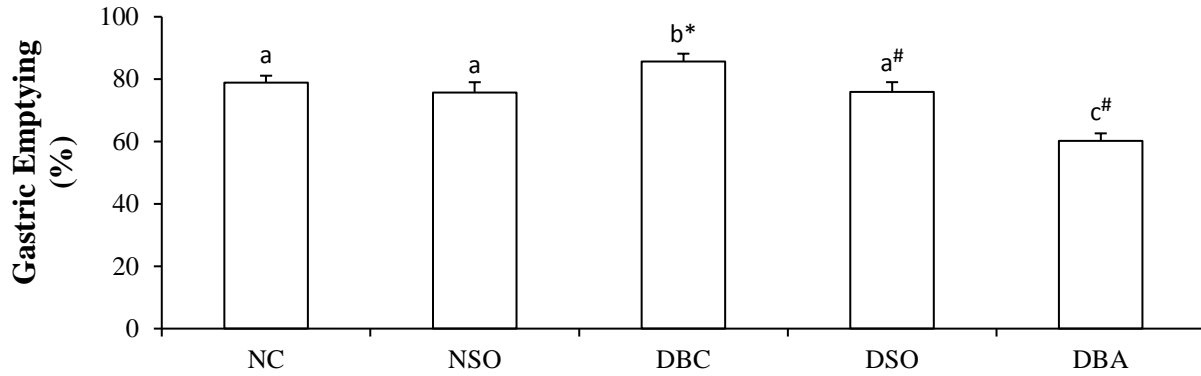
Figure 5.3: Effect of sorbitol on gastric emptying in different animal groups at the end of a 1 h experimental period. Data are presented as mean ± SD of 5–6 animals. [abc]Different letters presented above the bars are significantly different from each other group of animals, *significantly different from NC group and #significantly different from DBC group ($p < 0.05$; Tukey's HSD post-hoc test, IBM, SPSS version 21).NC, normal control; NSO, normal sorbitol; DBC, diabetic control; DSO, diabetic sorbitol, DBA, diabetic acarbose.
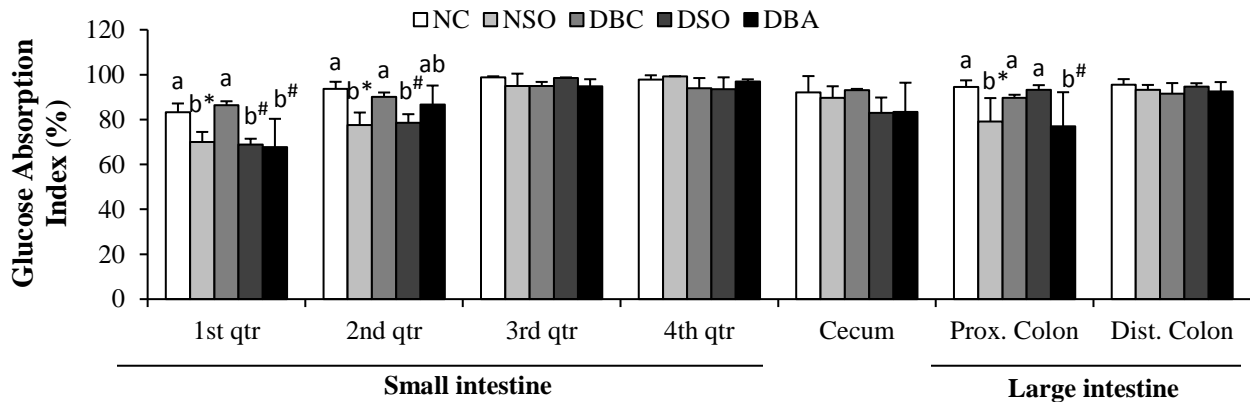
Figure 5.4: Effect of sorbitol on glucose absorption index (GAI) in the different GIT segments of different animal groups at the end of a 1 h experimental period. Data are presented as mean ± SD of five to six animals. [ab]Different letters presented above the bars for a given segment are significantly different from each other group of animals, *significantly different from NC group and #significantly different from DBC group ($p < 0.05$; Tukey's HSD post-hoc test, IBM, SPSS version 21). NC, normal control; NSO, normal sorbitol; DBC, diabetic control; DSO, diabetic sorbitol, DBA, diabetic acarbose.

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The single oral dose of sorbitol and acarbose co-ingested with glucose significantly reduced ($p > 0.05$) blood glucose increase 1 h after the dose administration in diabetic animals, although the results were not significantly different for normal animals (**Fig. 5.5**).
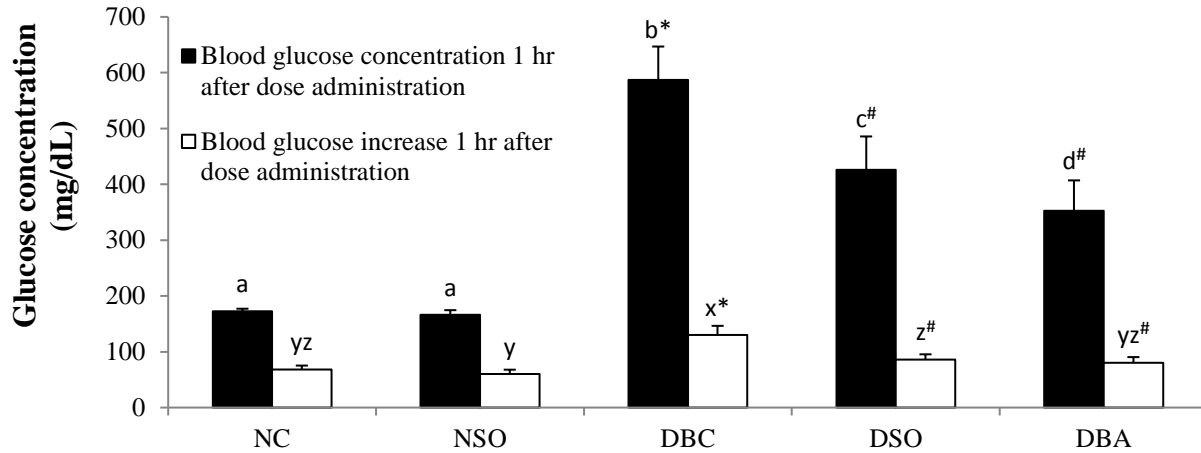

Figure 5.5: Effects of sorbitol on final blood glucose concentrations and blood glucose increase in animals after 1 h of the dose administration. Data are presented as mean ± SD of six animals. [a-d & x-z]Different letters presented above the bars are significantly different from each other group of animals, *significantly different from NC group and #significantly different from DBC group ($p < 0.05$; Tukey's HSD multiple range post-hoc test, IBM, SPSS version 21). NC, normal control; NMI, normal myo-inositol; DBC, diabetic control; DMI, diabetic myo-inositol; DBA, diabetic acarbose.

On the other hand sorbitol treatments significantly increased ($p < 0.05$) digesta transit from the 3rd qtr of the small intestine up to proximal colon in normal rats (**Table 5.2**). In diabetic animals however, sorbitol and acarbose treatment only significantly increased ($p < 0.05$) digesta transit in the cecum and proximal colon, while no significant difference was observed in the other intestinal segments regardless of the animal group or treatment (**Table 5.2**).

Table 5.2: Effect of sorbitol ondigesta transit in the different segments of the intestinal tract during 1 h experimental period

Group	1st qtr	2nd qtr	3rd qtr	4th qtr	Cecum	Prox. Colon
			Small Intestine			Large Intestine
			Digesta Transit [%]			
NC	95.26 ± 4.01[ab]	89.36 ± 4.58	70.27 ± 12.07[a]	48.53 ± 4.10[a]	23.68 ± 5.63[a]	36.81 ± 9.21[a]
NSO	96.46 ± 1.74[a]	90.67 ± 1.34	82.68 ± 3.70[b*]	73.93 ± 2.20[b*]	61.61 ± 4.69[b*]	68.16 ± 2.16[b*]
DBC	90.97 ± 0.08[b]	87.86 ± 1.59	76.55 ± 5.16[ab]	72.01 ± 7.04[b*]	37.56 ± 3.58[c*]	49.69 ± 0.90[c*]
DSO	95.89 ± 1.20[ab]	87.37 ± 3.88	78.43 ± 3.56[ab]	73.04 ± 5.52[b]	45.98 ± 5.41[d#]	62.98 ± 2.59[b#]
DBA	93.85 ± 4.72[ab]	87.01 ± 5.29	79.48 ± 6.09[ab]	71.76 ± 5.38[b]	65.26 ± 5.25[b#]	87.63 ± 3.07[d#]

Data are presented as mean ± SD of five to six animals. [abcd]Different superscript letters presented in each column for a given segment are significantly different from each other group of animals, *significantly different from NC group and #significantly different from DBC group ($p < 0.05$; Tukey's HSD post-hoc test, IBM, SPSS, version 21). GRP, Group; NC, normal control; NSO, normal sorbitol; DBC, diabetic control; DSO, diabetic sorbitol, DBA, diabetic acarbose.

5.5   Discussion

Sugar alcohols are known for their lower caloric value compared to sucrose when sorbitol is widely used due to its number of beneficial effects on health (Hayes, 2001; Livesey, 2003). It has been speculated that sorbitol may inhibit intestinal glucose absorption (WHO, 1987). A previous report suggested that sorbitol may be useful for glycemic control in normoglycemic and diabetic subjects (Akgün and Ertel, 1980), when a recent study reported that sorbitol possesses inhibitory effect on carbohydrate digesting enzyme activities *in vitro* (Kang *et al*., 2014). However, none of these previous studies investigated the effect of sorbitol on intestinal glucose absorption and muscle glucose uptake. Hence, the present study investigated the effects of sorbitol on intestinal glucose absorption and muscle glucose uptake to further understand the mechanisms behind the effects of sorbitol on dietary glucose absorption and uptake.

Dietary glucose is actively absorbed from the intestine, contributing to blood glucose rise, but absorption capacity of glucose varies from one intestinal segment to another. However, it has

been reported that majority of dietary glucose is likely to be absorbed from the proximal to mid small intestine (part of the duodenum and jejunum) (Bogner *et al*., 1963; Rider *et al*., 1967; Lavin, 1976), and for the same reason intestinal tissue from this section was used for the *ex-vivo* glucose absorption study. In our *ex vivo*study, the amount of glucose absorbed by the jejunal segments of rats reached a peak value of 5.20 ± 072 mg/cm jejunum after 2 hour incubation period in a glucose solution without sorbitol (**Fig. 5.1**). However, this peak value was significantly reduced ($p<0.05$) to 3.53 ± 0.50 mg/cm jejunum in the presence of 5% sorbitol and reached as low as 2.87 ± 0.70 mg/cm jejunum in the presence of 20% sorbitol, which suggests the possible inhibitory effect of sorbitol on intestinal glucose absorption.

Previous studies have demonstrated that co-ingestion of glucose with sorbitol increased sorbitol absorption relative to ingestion of sorbitol alone (Beaugerie *et al*., 1995; Beaugerie *et al*., 1996). Our *in vivo* data showed high glucose absorption index (GAI) across the different intestinal segments of rats (**Fig. 5.4**), which accounts for the complete and rapid intestinal absorption of glucose. However, the significantly reduced ($p<0.05$) GAI in the 1st and 2nd qtrs of the small intestine of normal and diabetic rats fed with sorbitol (**Fig. 5.4**) correspond to the results of our *ex vivo* glucose absorption study (**Fig. 5.1**) and further confirms the inhibitory potentials of sorbitol on intestinal glucose absorption. In fact, the observed inhibitory effect of sorbitol in the proximal small intestine is of particular interest, because the proximal small intestine is most likely the site where majority of glucose absorption occurs (Rider *et al*., 1967). In our study, sorbitol significantly inhibited the glucose absorption in this region of the small intestine, and the effect was comparable to that of a widely used anti-diabetic drug (acarbose) in both normal and diabetic animals (**Fig. 5.4**). Additionally, the reduced postprandial blood glucose increase observed in the sorbitol-fed animals (**Fig. 5.5**) may also be a reflection of the inhibitory effect of sorbitol on intestinal glucose absorption (**Fig. 5.4**). Based on the above discussions we hypothesize that the observed reduced intestinal glucose absorption in sorbitol-fed rats (**Fig. 5.4**) might be due to the reported increased intestinal sorbitol absorption when co-ingested with glucose (Beaugerie *et al*., 1995; Beaugerie *et al*., 1996). However, this hypothesis requires further investigation to elucidate the actual absorption-related interaction between sorbitol and glucose in the different sections of intestine.

Delayed gastric emptying and accelerated digesta transit can contribute to reduced intestinal nutrient absorption as well as food intake (Salminen *et al*., 1984; Shafer *et al*., 1987) and has been suggested to be partly involved in the mode of action of acarbose in reducing intestinal glucose absorption (Ranganath *et al*., 1998). In our study, the induction of diabetes significantly increased gastric emptying rate (**Fig. 5.3**), which depicts the clinical presentation of gastric emptying in most type 2 diabetic patients (Phillips *et al*., 1991; Horowitz *et al*., 1996. However, an oral bolus dose of sorbitol significantly reduced ($p<0.05$) gastric emptying in diabetic rats (**Fig. 5.3**) and increased the digesta and colonic transit in both normal and diabetic animals (**Table 5.2**). These data are in line with the results of previous studies in healthy human subjects (Hunt, 1963; Skoog *et al*., 2006) and may also contribute to the lower GAI in the proximal half of the small intestine (**Fig. 5.4**).

Under physiological condition, the blood glucose homeostasis is constantly maintained by the action of two important hormones: insulin and glucagon. Among other functions, insulin promotes the clearance of circulating glucose for either storage or energy production in cells, thus prevent abnormal increment of blood glucose level (Aronoff *et al*., 2004). Previous studies have reported the glycogenic effects of oral or parenteral sorbitol administration in rats (Stetten and Stetten, 1951), which suggests possibilities of sorbitol to enhance clearance of circulating glucose towards glycogen synthesis. In our present study, sorbitol exhibited a concentration-dependent increase in muscle glucose uptake with or without insulin compared to control (**Fig. 5.2**). Insulin also significantly enhanced the muscle glucose uptake effect of sorbitol (**Table 5.1**), which has also been observed previously (Turban *et al.* 2012; Kristensen *et al*., 2014). These data suggest the potential of sorbitol in improving insulin-mediated muscle glucose uptake at least in *ex vivo* condition, but still requires further investigation at *in vivo* level.

In summary, apart from the effects of sorbitol on enhancing muscle glucose uptake *ex vivo* (**Fig. 5.2** and **Table 5.1**), the results of our present study suggest that sorbitol possesses inhibitory effects on intestinal glucose absorption *ex vivo* (**Fig. 5.1**) which have been further supported by the results of the *in vivo* study in normal and diabetic rats (**Fig. 5.4** and **5.5**). Additionally, our present study also revealed that the ingestion of sorbitol delayed gastric emptying (**Fig. 5.3**) and accelerated digesta transit (**Table 5.2**) in normal and diabetic rats, which might partly contribute to the observed *in vivo* inhibitory effects sorbitol on small intestinal

glucose absorption (**Fig. 5.4**). Hence, sorbitol may be further investigated as a potential anti-hyperglycemic sweetener in diabetic foods and food products, which may ameliorate hyperglycemia via inhibiting intestinal glucose absorption and possibly enhancing muscle glucose uptake.

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CHAPTER 6

ERYTHRITOL REDUCES SMALL INTESTINAL GLUCOSE ABSORPTION, INCREASES MUSCLE GLUCOSE UPTAKE, IMPROVES GLUCOSE METABOLIC ENZYMES ACTIVITIES AND INCREASES mRNA EXPRESSION OF GLUT-4 AND IRS-1 IN TYPE 2 DIABETIC RATS

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Preface: This article reported the effects of erythritol on intestinal glucose absorption and muscle glucose uptake in normal and type 2 diabetic rats as possible underlying mechanisms behind its anti-hyperglycemic potentials. This article is under consideration for acceptance in Molecular Nutrition and Food Research, pending submission of revision that satisfies both reviewer and editorial team (manuscript number: mnfr.201601105).

6.1   Abstract

The present report investigated the effects of erythritol on intestinal glucose absorption and muscle glucose uptake as possible underlying mechanisms behind its anti-hyperglycemic potentials. Experiment 1 examined the effects of increasing concentrations (2.5 – 20%) of erythritolon glucose absorption and uptake in isolated rat jejunum and psoas muscle respectively. Experiment 2 examined the effects of a bolus dose of erythritol (1 g/kg bw) on intestinal glucose absorption, gastric emptying and postprandial blood glucose increase in normal and type 2 diabetic rats 1 h after the co-ingestion of erythritol with glucose (2 g/kg bw). Study 3 investigated the effects of a bolus dose of erythritol (1 g/kg bw) on glucose tolerance, serum insulin level, muscle/liver hexokinase and liver glucose-6 phospahtase activities and mRNA expression of muscle Glut-4 and IRS-1 in normal and type 2 diabetic animals. Study 1 revealed that erythritol dose-dependently enhanced muscle glucose uptake with or without insulin. Study 2 demonstrated that oral erythritol treatment delayed gastric emptying and reduced small intestinal glucose absorption as well as postprandial blood glucose rise, especially in diabetic animals. Study 3 showed that oral erythritol treatment improved glucose tolerance, insulin secretion, muscle/liver hexokinase and liver glucose-6 phospahtase activities and mRNA expression of muscle Glut-4 and IRS-1 in diabetic animals. Data of this study suggest that erythritol may exert anti-hyperglycemic effects not only via reducing small intestinal glucose absorption, but also by increasing muscle glucose uptake and improving glucose metabolic enzymes activity and Glut-4 and IRS-1 mRNA expression. Hence, erythritol may be a useful dietary supplement for managing hyperglycemia, particularly for type 2 diabetes (T2D).

6.2   Introduction

The increasing global socioeconomic threat caused by type 2 diabetes (T2D) has been linked to its higher prevalence compared to other types of diabetes. T2D accounts for more than 90% of total diabetes cases worldwide (Pratley, 2013) and currently one of the major threats to global public health (International Diabetes Federation, 2015). Unfortunately, almost 50% of people living with T2D are undiagnosed (Bahrami & Gerstein, 2016). T2D is described as a multifaceted and progressive disease that is linked to several metabolic defects and organ

malfunction or damage (Pratley, 2013). Insulin resistance and impairment of pancreatic beta cell function are the most prominent pathogenic features of T2D, which progressively results in defective insulin secretion and persistent hyperglycemia (Pratley & Weyer, 2001; Kahn, 2003). Insulin resistance in peripheral tissues, such as adipose and muscle tissues downregulates the signalling of cellular glucose uptake, which contributes to hyperglycemia in T2D (Pratley & Weyer, 2001; Kahn, 2003).

Furthermore, there are several risk factors that strongly influence the pathogenesis and progression of T2D. Sedentary life style, routine consumption of high fat and high calorie diets as well as chronic consumption of refined and simple carbohydrates like sucrose and fructose have been reported to be among the most influential factors in the progression of T2D (Thorburn *et al*., 1989; Elliott *et al*., 2002; Astrup *et al*., 2002; Hu, 2003). In a previous study, it has been reported that 10 weeks consumption of sucrose equivalent to 28% of dietary energy increased food intake, body weight, body fat mass, and blood pressure compared to the consumption of non-sucrose sweeteners in overweight humans (Astrup *et al*., 2002). Furthermore, Thorburn *et al*. (1989) reported that the consumption of fructose equivalent to 35% of energy requirement reduced glucose disposal and insulin action in the liver and most peripheral tissues. Accordingly, dietary adjustments limiting the intake of added sugars, especially in diabetic individuals have been greatly encouraged (Amod *et al*., 2012). Consequently, sugar substitutes including non-nutritive sweeteners such as saccharin, aspartame, stevia, sucralose, neotame etc and sugar alcohols such as xylitol, sorbitol, maltitol, erythritol, mannitoletc have become part of diabetic and non-diabetic diets, because of their minimal effect in increasing postprandial blood glucose and their lower caloric value compared to easily digestible simple carbohydrates such as glucose, sucrose and fructose (Livesey, 2003; Fitch & Keim, 2012). Sugar alcohols are known for their wide application, including oral health care application (Mäkinen, 2010) and erythritol is one of the popular sugar alcohols in the market.

Erythritol, a four-carbon sugar alcohol, is a very unique sugar alcohol not only due to its zero caloric value and no glycemic effects, but also due to its minimal gastrointestinal side effects compared to other sugar alcohols (Livesey, 2003). Although it is well absorbed from the small intestine compared to other sugar alcohols (about 90% of ingested amount), it is not metabolized (Livesey, 2003) but completely excreted in the urine, thus does not contribute to the

systemic glucose pool. Apart fromthe zero caloric and glycemic effects, a number of studies have suggested the anti-hyperglycemic potentials of erythritol.

In a previous study, Bornet *et al*. (1996) reported that the mean levels of plasma glucose and insulin in healthy humans remained unaffected up to 3 h after a single oral dose of 1 g/kg bw of erythritol (Bornet *et al*., 1996). In diabetic rats, 10 days oral administration of 100 to 400 mg/kg bw of erythritol significantly reduced blood glucose level as well as oxidative stress markers like protein glycosylation and lipid peroxidation in the serum, liver and kidney (Yokozawa *et al*., 2002). Fourteen days oral administration of 20 g erythritol per day continuously reduced serum glucose and glycated hemoglobin (HbA1c) in human subjects with non-insulin dependent diabetes mellitus (Ishikawa *et al*., 1996). Furthermore, in a recent study, Flint *et al*. (2014) reported that short term (4 weeks) oral administration of erythritol (30 g/day) improved arterial endothelial function in type 2 diabetic subjects (Flint *et al*., 2014), which suggests that erythritol may be useful in the management of diabetes-related microvascular complications. In a most recent study, it was reported that a single dose of 75 g erythritol in 300 ml of water, supplied via nasogastric tube modulated gut hormone (GPL-1) and also delayed gastric emptying in lean and obese normoglycemic subjects (Woelnerhanssen *et al*., 2016). Glucose tolerance was also not significantly affected in the erythritol fed group compared to control group (normal water feeding) of this study, which suggests that erythritol could be useful in the control of postprandial glucose elevation.

Despite the above-mentioned anti-hyperglycemic potentials of erythritol in normoglycemic and diabetic conditions, further investigation are still required to understand the underlying mechanisms and mode of actions behind its anti-hyperglycemic effects. Hence, this study was conducted to investigate the effects of erythritol on intestinal glucose absorption, muscle glucose uptake and on the activities of related enzymes and expression of related genes using *ex vivo* and *in vivo* experimental models.

6.3   Materials and methods

Please refer to chapter 2, sub-sections 2.2.1 and 2.2.3 pages 66-83 for detailed material and methods of this article.

6.4 Results

6.4.1 *Ex vivo* study (experiment 1)

Effects of erythritol on intestinal glucose absorption and muscle glucose uptake

Data for the effects of erythritol on intestinal glucose absorption and muscle glucose uptake are presented in **Fig. 6.1** and **6.2** as well as **Table 6.1**. Erythritol did not significantly influence glucose absorption in isolated rat jejunum (**Fig. 6.1**). On the other hand, erythritol caused a concentration-dependent increase of glucose uptake in isolated psoas muscle with or without insulin (**Fig. 6.2**). Muscle glucose uptake of all concentrations of erythritol and 2 mg/ml metformin was significantly higher ($p<0.05$) than the control, while glucose uptake of 2 mg/ml metformin did not differ from glucose uptake of 10% and 20% erythritol (**Fig. 6.2**). Insulin significantly enhanced the muscle glucose uptake effect of erythritol; the computed GU_{50} of erythritol was 2.02 ± 1.24 % with insulin and 6.91 ± 2.03 % without insulin (**Table 6.1**).
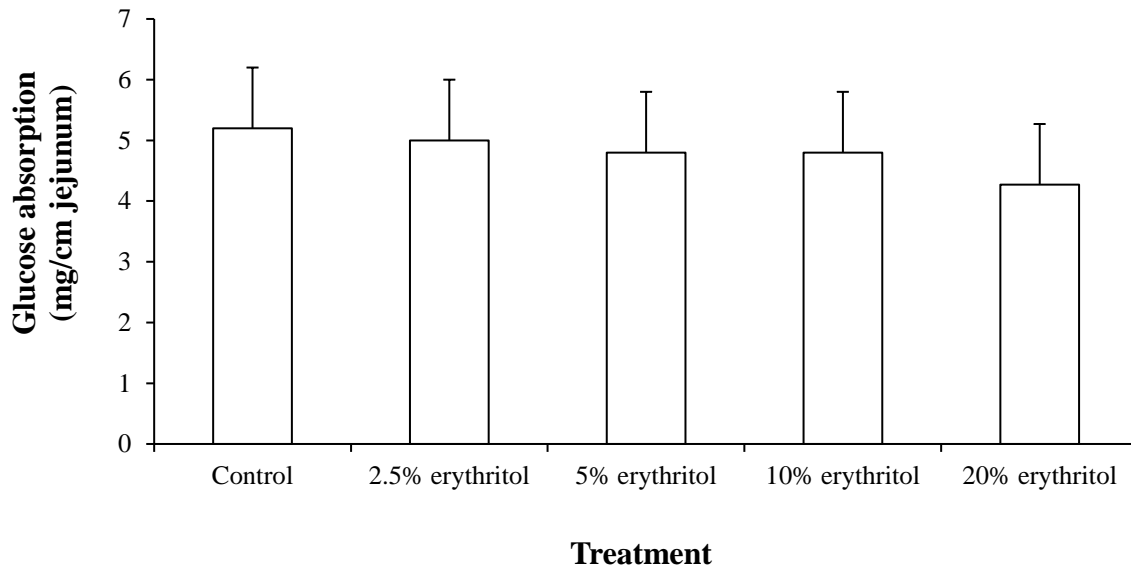

Figure 6.1: Effect of erythritol on glucose absorption in isolated rat jejunum. Data are presented as mean $\pm$ SD of five replicates of analysis ($p < 0.05$; Tukey's HSD multiple range post-hoc test, IBM, SPSS version 21).
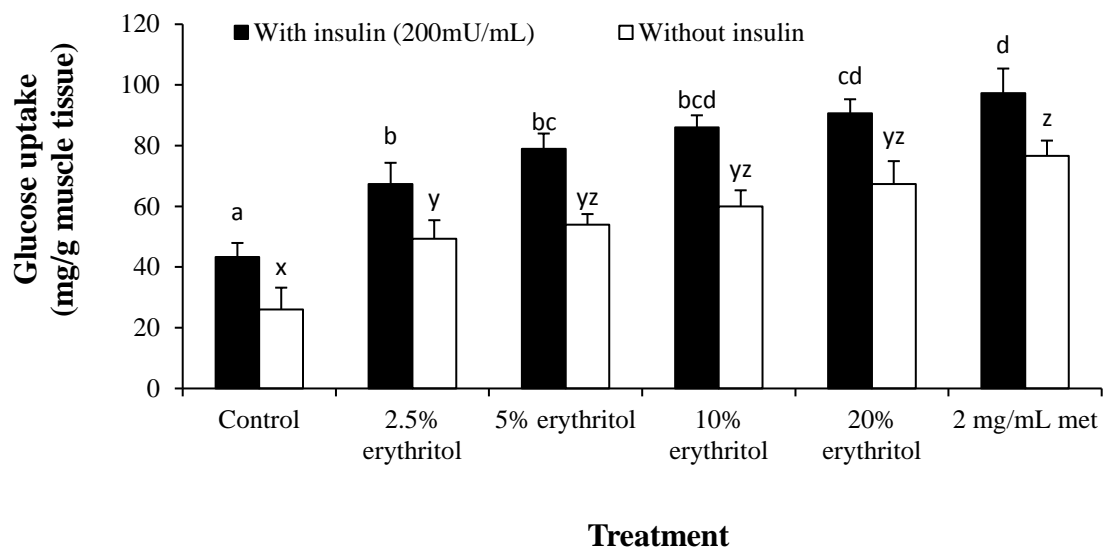
Figure 6.2: Effect of erythritol on glucose uptake with or without insulin in isolated rat psoas muscle. Data are presented as mean ±SD of triplicate analysis. [a-d or x-z] Different letters presented above the bars for with or without insulin are significantly different from each other ($p < 0.05$; Tukey's HSD multiple range post-hoc test, IBM, SPSS version 21).

Table 6.1: Percentage GU_{50} values of glucose uptake increase of erythritol in isolated psoas muscle, with and without insulin

	With insulin (200mU/mL)	Without insulin
GU_{50} (%)*	2.02 ± 1.24^a	6.91 ± 2.03^b

Data are presented as mean ± SD of triplicate analysis. [ab]Values with different superscript letters are significantly different from each other ($p < 0.05$; Tukey's HSD multiple range *post-hoc* test, IBM, SPSS version 21). *GU_{50}: Concentration (% as w/v) of erythritol required to cause 50% increase of muscle glucose uptake.

Additionally, muscle tissue viability studies confirmed that 1 h incubation of psoas muscle tissue did not significantly affect the viability of the tissue (Please refer to **Table 3.2** in chapter 3, sub-section 3.4.1, section 3.4, page 92 for supporting data).

6.4.2 *In vivo* intestinal glucoseabsorption study (experiment 2)

Effects of erythritol on gastric emptying, glucose absorption index and blood glucose level

The data for the effects of erythritol on gastric emptying, glucose absorption index and blood glucose level are presented in **Fig. 6.3** to **6.5**. Erythritol significantly ($p<0.05$) delayed gastric emptying in normal animals (**Fig. 6.3**). Induction of diabetes significantly ($p<0.05$) accelerated gastric emptying, while erythritol and acarbose significantly reduced diabetes-induced accelerated gastric emptying with statistically similar capacity (**Fig. 6.3**).
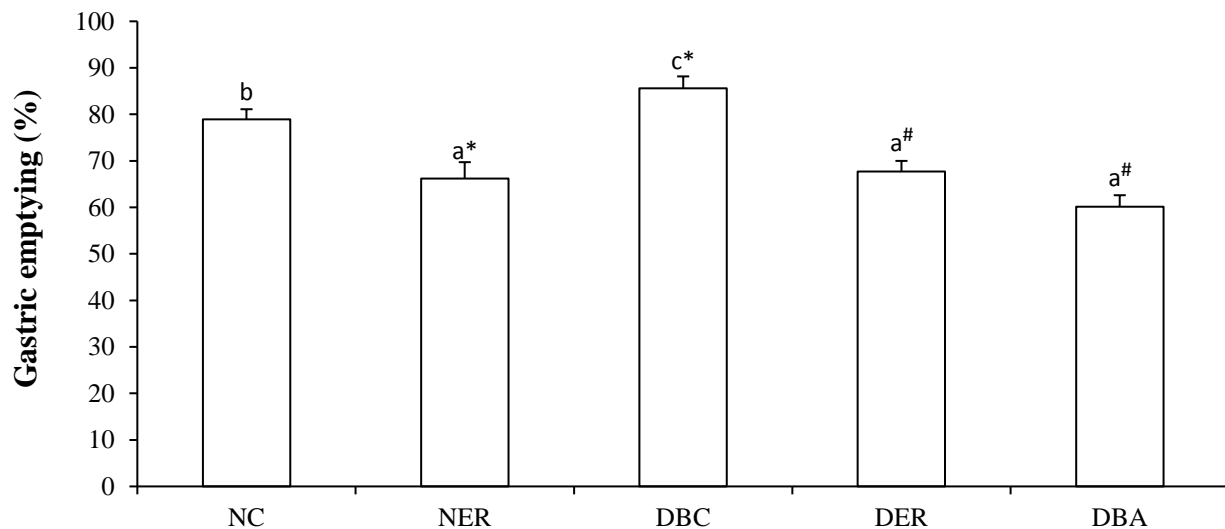

Figure 6.3: Effect of erythritol on gastric emptying in different animal groups at the end of a 1 h experimental period. Data are presented as mean ± SD of 5–6 animals. [abc]Different letters presented above the bars are significantly different from each other group of animals, *significantly different from NC group and [#]significantly different from DBC group ($p<0.05$; Tukey's HSD post-hoc test, IBM, SPSS version 21).NC, normal control; NER, normal erythritol; DBC, diabetic control; DER, diabetic erythritol; DBA, diabetic acarbose.

Furthermore, erythritol significantly reduced (p<0.05) GAI in the 1st qtr of the small intestine of normal and diabetic animals, while acarbose significantly reduced (p<0.05) GAI in the 1st qtr of the small intestine and also in the proximal colon of diabetic animals (**Fig. 6.4**).
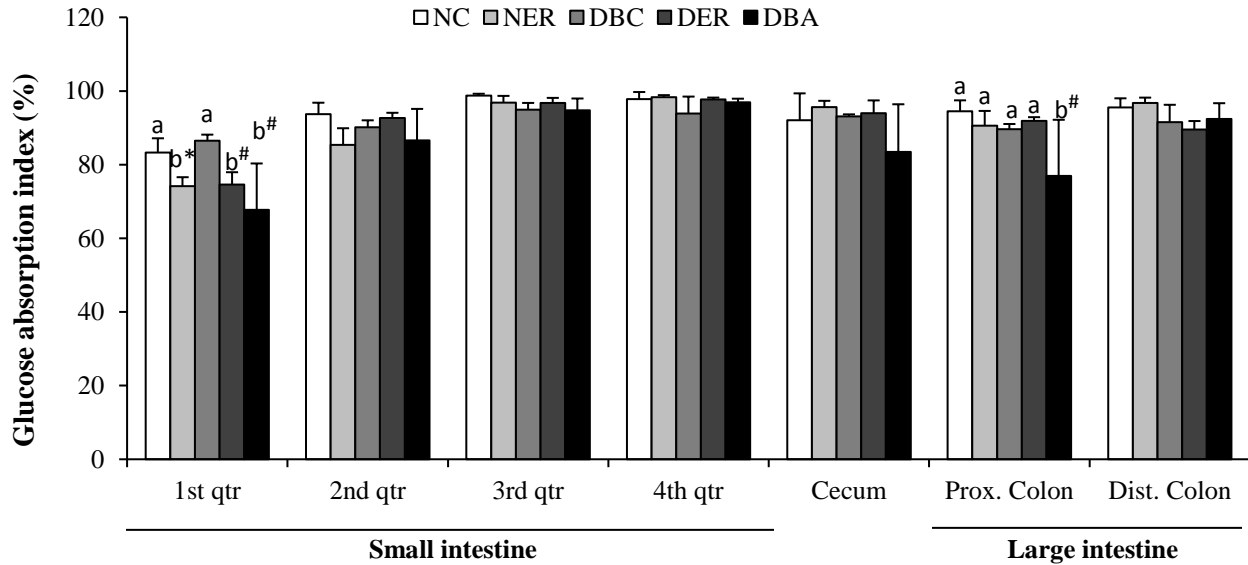

Figure 6.4: Effect of erythritol on glucose absorption index (GAI) in the different GIT segments of different animal groups at the end of a 1 h experimental period. Data are presented as mean ± SD of five to six animals. [ab]Different letters presented above the bars for a given segment are significantly different from each other group of animals, *significantly different from NC group and #significantly different from DBC group (p<0.05; Tukey's HSD post-hoc test, IBM, SPSS version 21). NC, normal control; NER, normal erythritol; DBC, diabetic control; DER, diabetic erythritol; DBA, diabetic acarbose.

On the other hand, erythritol did not significantly influence blood glucose increase in normal animals, while in diabetic animals, erythritol and acarbose significantly prevented (p<0.05) blood glucose increase compared to diabetic control group (**Fig. 6.5**).
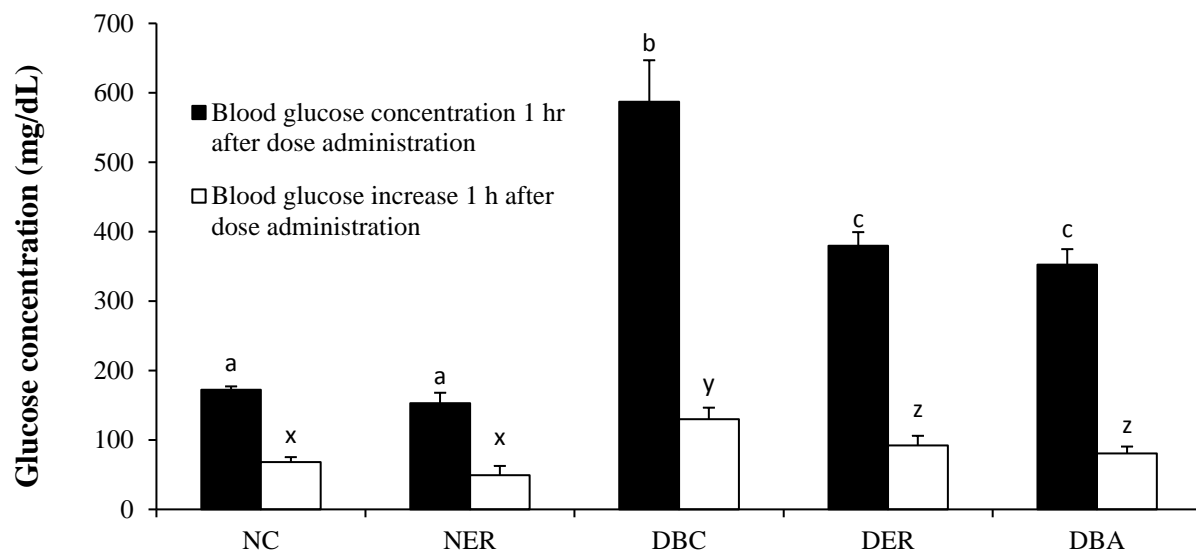
Figure 6.5: Effect of erythritol on final blood glucose concentrations and blood glucose increase in animals after 1 h of the dose administration. Data are presented as mean ± SD of six animals. [a-d & x-z]Different letters presented above the bars are significantly different from each other group of animals, *significantly different from NC group and #significantly different from DBC group (p<0.05; Tukey's HSD multiple range post-hoc test, IBM, SPSS version 21). NC, normal control; NER, normal erythritol; DBC, diabetic control; DER, diabetic erythritol; DBA, diabetic acarbose.

6.4.3 *In vivo* muscle glucose uptake study (experiment 3)

Effects of erythritol on oral glucose tolerance and serum insulin level

The data for oral glucose tolerance test and serum insulin are presented in **Fig. 6.6** and **6.7**. Although erythritol did not significantly influence the glucose tolerance ability of normal animals, it significantly improved (p<0.05) the glucose tolerance ability of diabetic animals, especially at 30 and 60 min after the glucose ingestion (**Fig. 6.6**). On the other hand, erythritol did not significantly influence serum insulin levels in normal animals, but it appreciably increased (p = 0.178) diabetes-induced serum insulin depletion of diabetic animals, although the data were not significantly different, perhaps due to high standard deviations (**Fig. 6.7**).
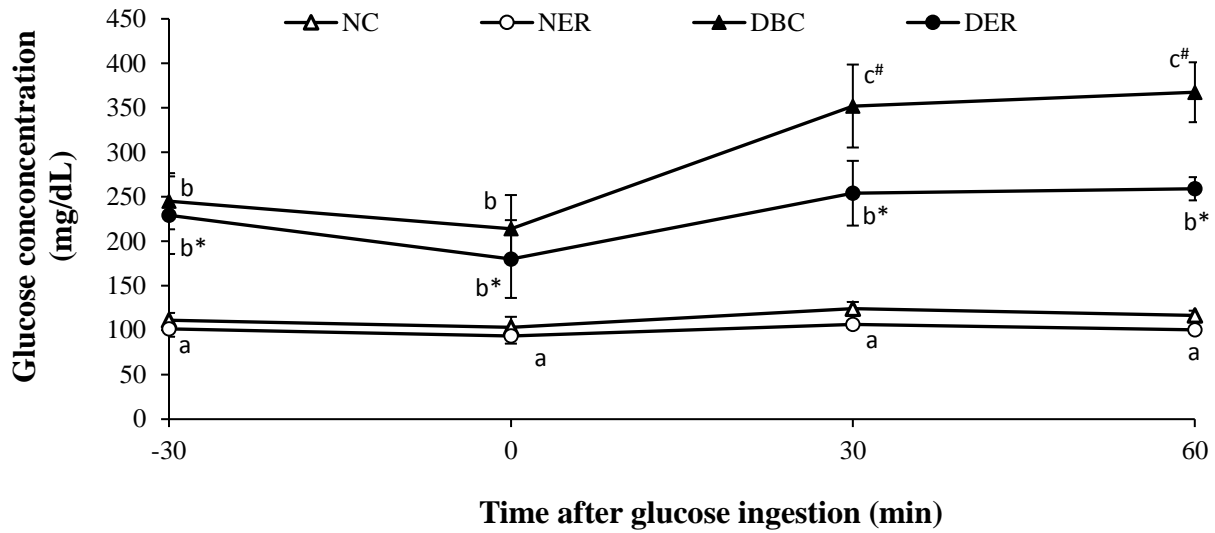
Figure 6.6: Effect of erythritol onoral glucose tolerance. Data are presented as mean ± SD of four animals. "abc"Different letters presented above the bars are significantly different from each other group of animals,*significantly different from NC group and #significantly different from DBC group (p<0.05; Tukey's HSD multiple range post-hoc test, IBM, SPSS version 23). NC, normal control; NER, normal erythritol; DBC, diabetic control; DER, diabetic erythritol.
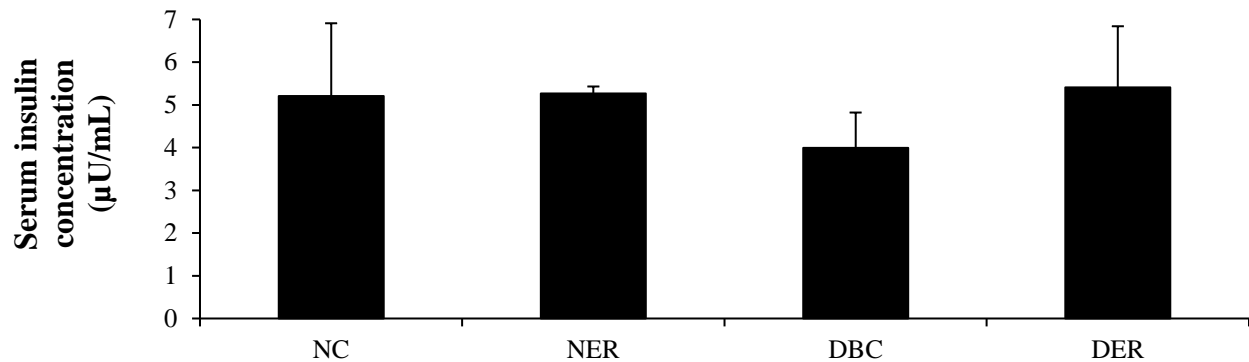

Figure 6.7: Effect of erythritol on serum insulin level. Data are presented as mean ± SD of four animals. "abc"Different letters presented above the bars are significantly different from each other group of animals,*significantly different from NC group and #significantly different from DBC group (p<0.05; Tukey's HSD multiple range post-hoc test, IBM, SPSS version 23). NC, normal control; NER, normal erythritol; DBC, diabetic control; DER, diabetic erythritol.

Effects of erythritol on muscle hexokinase, liver glucokinase and liver glucose-6 phosphates activities

The data for muscle hexokinase, liver glucokinase and liver glucose-6-phosphatase activities are presented in **Fig. 6.8**. Erythritol did not significantly influence the activities of muscle hexokinase (**Fig. 6.8a**), liver glucokinase (**Fig. 6.8b**) and liver glucose-6 phosphatase (**Fig. 6.8c**) in normal animals. Diabetes induction significantly reduced ($p<0.05$) muscle hexokinase and liver glucokinase activities and significantly increased ($p<0.05$) liver glucose-6 phosphates activity. While erythritol markedly improved diabetes-induced reduction of muscle hexokinase ($p = 0.075$) and liver glucokinase ($p = 0.058$) activities (**Fig. 6.8a and 6.8b**), it significantly reduced ($p<0.05$) diabetes-induced elevation of glucose-6 phosphates activity in the liver (**Fig. 6.8c**).
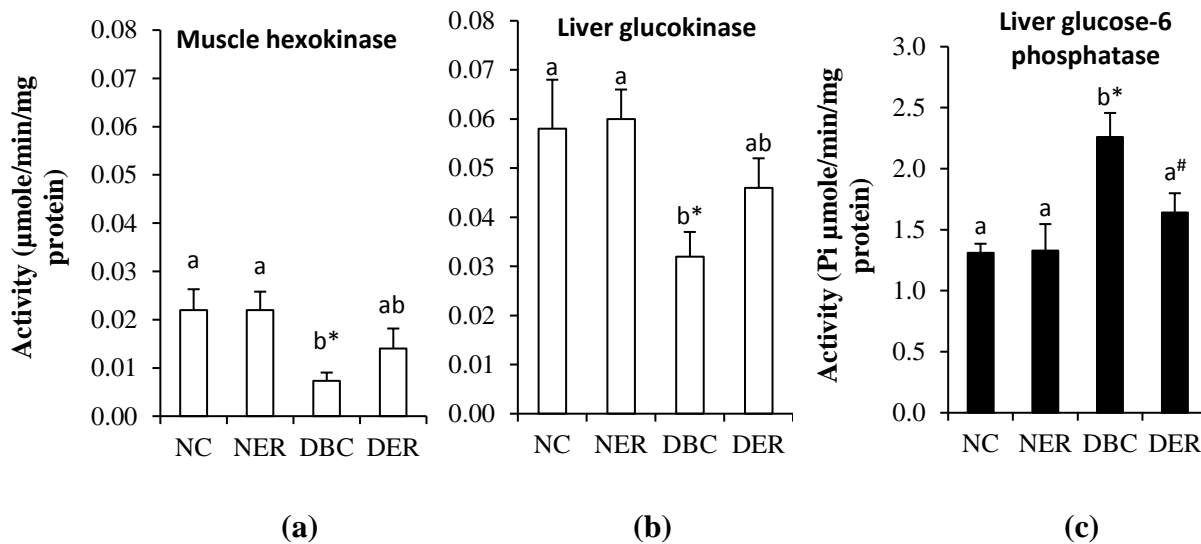

(a)   **(b)**   **(c)**

Figure 6.8: Effects of erythritol on **(a)** muscle hexokinase,**(b)** liver glucokinase and **(c)** liver glucose-6 phosphatase activities.Data are presented as mean ± SD of four animals. "[abc]"Different letters presented above the bars are significantly different from each other group of animals,*significantly different from NC group and [#]significantly different from DBC group ($p<0.05$; Tukey's HSD multiple range post-hoc test, IBM, SPSS version 23). NC, normal control; NER, normal erythritol; DBC, diabetic control; DER, diabetic erythritol.

Effects of erythritol on mRNA expression of muscle Glut-4 and IRS-1

The data for the mRNA expression muscle Glut-4 and IRS-1 are presented in Fig. 6.9. Gel image for β-Actin showed that there was no notable change in the mRNA expression of the reference gene across all animal groups (**Fig. 6.9a** and **Supplementary data 6**), hence it was suitable for normalizing the expression of our genes of interest. Erythritoltreatment did not significantly influence the mRNA expression of muscle Glut-4 and IRS-1 in normal animals (**Fig. 6.9a** and **6.9b**). Induction of diabetes decreased ($p<0.05$) the mRNA expression of muscle Glut-4 and IRS-1 by 10.39 ± 1.92 folds and 3.56 ± 0.61 folds, respectively (**Fig. 6.9b**). However, decreased Glut-4 and IRS-1 mRNA expression of diabetic animals increased ($p<0.05$) by 16.14 ± 2.23 folds and 4.80 ± 0.65 folds, respectively after erythritol treatment (**Fig. 6.9b**).
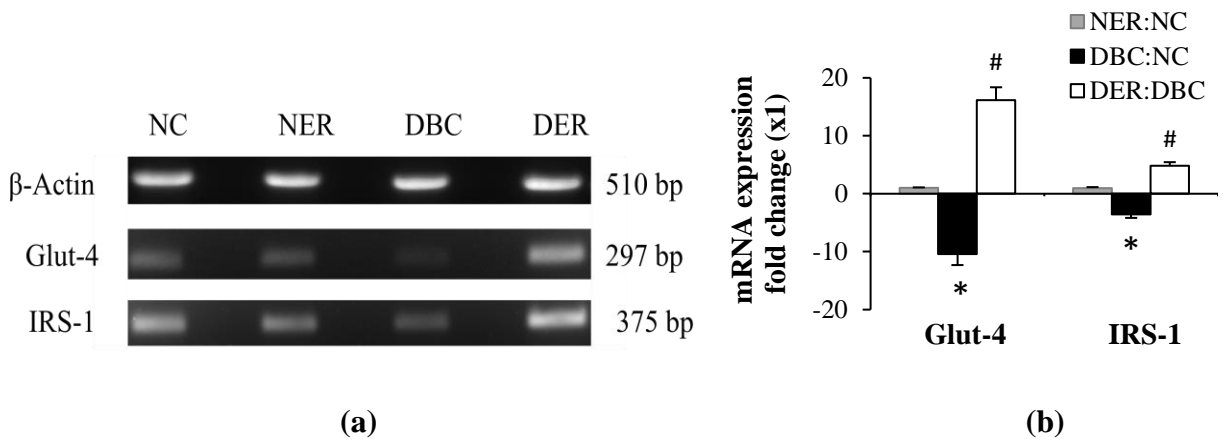

(a) (b)

Figure 6.9: Effect of erythritol on Glut-4 and IRS-1 mRNA expression. **(a)** qPCR products of target genes subjected to a 2% agarose gel electrophoresis and product sizes estimated with a 1 kb plus ladder (Refer to **Supplementary data 6** for complete gel image with 1 kb plus ladder). NC, normal control; NER, normal erythritol; DBC, diabetic control; DER, diabetic erythritol. **(b)** Fold changes in mRNA expressions of Glut-4 and IRS-1 due to erythritol treatment in normal animals (NER:NC); due to induction of diabetes in normal animals (DBC:NC); and due to erythritol treatment in diabetic animals (DER:DBC). Data are presented as mean ± SD of n = 4.* and # different symbols means mRNA expression fold change of Glut-4 and IRS-1 are statistically significant ($p < 0.05$; Tukey's HSD multiple range *post-hoc* test, IBM, SPSS version 23).

6.5 Discussion

The zero caloric as well as no glycemic effect of erythritol is a unique property compared to other sugar alcohols and a desirable quality for the management of T2D (Livesey, 2003). Additionally, erythritol has shown promising anti-hyperglycemic potentials in both normoglycemic (Bornet *et al*., 1996; Woelnerhanssen *et al*., 2016) and diabetic (Ishikawa *et al*., 1996; Yokozawa *et al*., 2002) human subjects, which supports its effects on ameliorating several T2D-related metabolic defects (Ishikawa *et al*., 1996; Flint *et al*., 2014). Our present study revealed underlying mechanisms of action behind the anti-hyperglycemic effects of erythritol in normal and type 2 diabetic conditions.

Postprandial glucose level is strongly influenced by the degree of glucose absorption in the small intestine, especially at the proximal region (jejunal and duodenal regions), which has been reported as the highest glucose absorption site of the small intestine (Rider *et al*., 1967; Riesenfeld *et al*., 1980). However, the rate of gastric emptying may also influence the extent of small intestinal nutrient absorption, including glucose absorption (Salminen *et al*., 1984), which can eventually influence postprandial glucose level (Aronoff *et al*., 2004). In fact, there are several evidences supporting the fact that accelerated gastric emptying could be one of the clinical metabolic defects contributing to hyperglycemia in diabetes subjects, especially type 2 (Phillips *et al*., 1991; Horowitz *et al*., 1996; Aronoff *et al*., 2004); and that delaying of gastric emptying could be a mode of action to reduce postprandial glucose increase in diabetes subjects (Ranganath *et al*., 1998). Although in our *ex vivo* study (experiment 1), erythritol did not significantly reduce glucose absorption in isolated rat jejunum (**Fig. 6.1**), our *in vivo* absorption study (experiment 2) revealed that oral erythritol treatment significantly reduced glucose absorption in the 1st qtr of the small intestine (**Fig. 6.4**) and concomitantly delayed (p<0.05) gastric emptying (**Fig. 6.3**) of both normal and diabetic animals. These data suggest that the delayed gastric emptying effect of erythritol (**Fig. 6.3**), which has also been recently observed in normoglycemic human subjects (Woelnerhanssen *et al*., 2016) could be a major contributing factor in its ability to decrease small intestinal glucose absorption in normal and diabetic animals (**Fig. 6.4**). Furthermore, reduced postprandial glucose rise was also observed in the erythritol-treated animals, especially in diabetic condition (p<0.05) (**Fig. 6.5**), which could be a reflection of the reduced small intestinal glucose absorption in similar group of animals (**Fig. 6.4**). These

results suggest the potency of erythtritol to improve or ameliorate hyperglycemia via inhibiting small intestinal glucose absorption.

On the other hand, blood glucose homeostasis under normal physiological state is constantly maintained by the action of two important hormones: insulin and glucagon. Insulin is the major hormone that up regulates peripheral glucose uptake via a cascade of signaling components, which favours systemic glucose disappearance (Aronoff *et al*., 2004). Insulin binds to the extracellular α-subunit of membrane-bound insulin receptor molecule (glycoprotein), which causes the activation of tyrosine kinase of the intracellular β-subunit (catalytic domain) (Chang *et al*., 2004). Activated tyrosine kinase then phosphorylates the insulin receptor substrate family, especially IRS-1 and IRS-2, which causes them to display binding sites for numerous signaling partners, such as phosphoinositide 3-kinase (PI3-K) to bind (Chang *et al*., 2004; González-Sánchez & Serrano-Ríos, 2007). PI3-K plays a major role in metabolic functions such as stimulating glucose uptake in peripheral tissues, especially muscle and fat tissues via a downstream cascade of events that leads to the activation of Akt (a serine/threonine protein kinase identified as one of the downstream targets of activated PI3-K) (González-Sánchez & Serrano-Ríos, 2007). Activated Akt signals the translocation of Glut-4 to the plasma membrane as well as the cellular influx of circulating glucose for cellular metabolic functions (Chang *et al*., 2004; González-Sánchez & Serrano-Ríos, 2007). A major cellular metabolic function of glucose is energy production via glycolysis-mediated metabolic pathways, especially in actively respiring cells of muscle tissues (Aronoff *et al*., 2004). Glycolytic pathway is initiated by a hexokinase-catalyzed glucose phosphorylation, which confers a negative charge on glucose to prevent glucose molecules entering cells from exiting, and thus maintaining muscle glucose uptake and systemic glucose disappearance (Wu *et al*., 2005).

Impaired muscle glucose uptake and systemic glucose disappearance during the development and progression of T2D are mainly associated with insulin resistance and subsequent impaired insulin secretion. This is caused by the dysfunction of cellular insulin signalling due to down regulation of major components of the insulin signalling pathway (Fröjdö*et al*., 2009; Boucher *et al*., 2014), including Glut-4 and IRS-1 (Carvalho*et al*., 2001; Wang *et al*., 2009; Kampmann*et al*., 2011). Hence, we investigated the effect of erythritol on muscle glucose uptake as a possible anti-hyperglycemic mechanism of action. Interestingly, data

of our *ex vivo* study (experiment 1) showed that erythritol dose-dependently increased ($p<0.05$) glucose uptake in isolated rat psoas muscle with or without insulin (**Fig. 6.2** and **Table 6.1**), which suggests that erythritol may potentiate insulin-mediated glucose uptake in muscles. This was further confirmed in the supporting data of our *in vivo* glucose uptake study (**experiment 3**), where acute oral erythritol treatment in diabetic animals appreciably improved Glut-4 and IRS-1 mRNA expression ($p<0.05$) (**Fig. 6.9a** and **6.9b**), serum insulin levels ($p = 0.178$) (**Fig. 6.7**) and oral glucose tolerance ($p<0.05$) (**Fig. 6.6**), which were initially reduced due to diabetes induction. Additionally, acute oral erythritol treatment concomitantly improved ($p = 0.075$) muscle hexokinase activities in diabetic animals (**Fig. 6.8a**), which demonstrates that erythritol improved glucose tolerance and metabolism via improving insulin secretion and modulating insulin-mediated muscle glucose uptake; thus, ameliorated hyperglycemia.

Furthermore, while insulin stimulates metabolic processes that favour systemic glucose disappearance, it also inhibits metabolic processes that can contribute to systemic glucose pool, especially when blood glucose rises above normal (Aronoff *et al*., 2004). This bi-functional role of insulin works in concert to ensure glucose homeostasis. Liver glycogenolysis is the major source of endogenous glucose contributing to systemic glucose pool, which is down regulated by insulin signalling when systemic glucose pool rises above normal (Aronoff *et al*., 2004). Liver glucose-6 phosphatase catalyzes the dephosphorylation of glucose-6 phosphate resulting from glycogenolysis, thus releasing glucose into circulation (Liu *et al*., 1994). On the other hand, liver glucokinase catalyzes the cellular phosphorylation of glucose, which helps to maintain the influx of glucose into hepatocytes for glycogen synthesis, thus enhancing systemic glucose disappearance (Lenzen, 2014). Both liver enzymes have been reported to be adversely affected during T2D, hence disrupting glucose homeostasis. In a previous study, it was reported that increased glucose-6 phosphatase activity and reduced glucokinase activity are major contributing factors of elevated endogenous glucose production in T2D (Clore *et al*., 2000). In another study, it was reported that induction of acute diabetes using streptozotocin significantly increased the activity and mRNA expression of hepatic microsomal glucose-6-phosphatase in young, juvenile and adult rats, aggravating hyperglycemia (Liu *et al*., 1994). In our present study, consistent trend was also observed, where induction of diabetes significantly increased liver glucose-6-phosphatase activity (**Fig. 6.8c**) and significantly reduced liver glucokinase activity (**Fig. 6.8b**). However, acute oral treatment with erythritol appreciably reversed this trend in diabetic animals

(Fig. 6.8c and 6.8b), which suggests that erythritol possesses the potency to improve glucose metabolism in diabetic conditions via enhancing systemic glucose disappearance to ameliorate hyperglycemia.

In summary, data from this study demonstrate the potency of erythritol in reducing small intestinal glucose absorption and enhancing insulin secretion and insulin-mediated muscle glucose uptake and metabolism via improving glucose metabolic enzyme activity and enhancing Glut-4 and IRS-1 mRNA expression, especially in diabetic animals. Although these anti-hyperglycemic effects of erythritol were investigated separately in separate studies, we propose that the bi-functional anti-hyperglycemic potency of erythritol (impairment of small intestinal glucose absorption and enhancement insulin-mediated muscle glucose uptake and metabolism) may work in concert to improve glucose tolerance and ameliorate hyperglycemia, especially in diabetic animals. Hence erythritol may be a useful dietary supplement for managing hyperglycemia in diabetic individuals, particularly for T2D.

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CHAPTER 7

GENERAL DISCUSSIONS AND CONCLUSIONS

7.1   General discussions

The inadequate attention given to diabetes mellitus, especially type 2, coupled with the unhealthy eating habits of people have aided in the rapid increase in the prevalence of diabetes and related diseases globally, including Africa. From the results of a recent survey, it has been reported that among the IDF geographical regions, Africa will have the highest percentage increase (140.8%) of people living with diabetes by 2040 (IDF, 2015). Considering the mortality rate of diabetes and the enormous expenditure on healthcare for people with diabetes (IDF, 2015), this projection will aggravate socioeconomic problems in the continent. Unfortunately, the dietary habit and lifestyle of most people has continuously stimulated the increasing trend of T2D and related diseases like obesity. In South Africa for example, the consumption of sugar or sugar-sweetened products, especially among adolescents is continuously increasing (Steyn & Temple, 2012), despite the fact that this is major a player in the development of T2D, obesity and metabolic syndrome (Elliott *et al*., 2002; Astrup *et al*., 2002; Hu & Malik, 2010; Stanhope, 2012). As an intervention strategy, there has been a strong campaign to control sugar intake especially in diabetics (Amod *et al*., 2012; Steyn & Temple, 2012). In this light, sweeteners or sugar substitutes such as sugar alcohols, including xylitol, sorbitol, maltitol, myo-inositol and erythritol are increasingly gaining public endorsement because of their lower calorie content compared to regular table sugar without sacrificing the sweetening appetite of people (Talbot & Fisher, 1978; Fitch & Keim, 2012). Despite the low caloric nature of some of these sugar alcohols, several studies have shown that they possess anti-hyperglycemic or hypoglycemic potentials (Akgün and Ertel, 1980; Vessby *et al*., 1990; Ishikawa *et al*., 1996; Santamaria *et al*., 2012; Rahman & Islam, 2014). Thus, this study was conducted to understand the mechanisms underlying the anti-hyperglycemic potentials of some of these commonly used sugar alcohols, which includes maltitol, myo-inositol, sorbitol and erythritol.

In order to achieve the set objectives, the afore-mentioned sugar alcohols were subjected to thorough *ex vivo* and *in vivo* investigations, examining their potency to exert glycemic control

via measuring their effects on intestinal glucose absorption or muscle glucose uptake. This is because intestinal absorption and muscle uptake of glucose are known therapeutic targets to ameliorate hyperglycemia in diabetics (Patel *et al.*, 2012).

Table 7.1: Summary of the *ex vivo* activities of tested sugar alcohols on the inhibition of intestinal glucose absorption and enhancement of muscle glucose uptake.

Activity (% w/v)	Sugar alcohols tested			
	Maltitol	Myo-inositol	Sorbitol	Erythritol
IC_{50}	3.57 ± 1.18	28.30 ± 6.01	14.46 ± 4.60	55.90 ± 11.98
GU50 with insulin	7.31 ± 2.08	2.68 ± 0.75	3.53 ± 1.61	2.02 ± 1.24
GU50 without insulin	111.12 ± 19.36	8.61 ± 0.55	7.02 ± 0.46	6.91 ± 2.03

Data are presented as mean ± SD of triplicate analysis. IC50: Concentration (% as w/v) of sugar alcohol required to cause 50% inhibition on intestinal glucose absorption.GU50: Concentration (% as w/v) of sugar alcohol required to cause 50% increase of muscle glucose uptake.

Maltitol was the first and only disaccharide sugar alcohol under study, which exhibited the highest inhibitory activity on intestinal glucose absorption *ex vivo*, but did not show remarkable glucose uptake activity on isolated rat psoas muscle with or without insulin (**Table 7.1**). In fact maltitol exhibited the least *ex vivo* muscle glucose uptake activity among all tested sugar alcohols (**Table 7.1**). These data suggest that maltitol may possess the potency to impair intestinal glucose absorption rather than enhancing muscle glucose uptake. In spite of this promising inhibitory activity of maltitol on intestinal glucose absorption *ex vivo*, bolus dose of maltitol (0.6 g/kg bw) did not affect intestinal glucose absorption or gastric emptying in both normal and diabetic animals (**experiment 2**). Accordingly, blood glucose level did not change significantly in both normal and diabetic animals after 1 h of a bolus maltitol feeding. Although these findings contradict our expectations, the poor intestinal glucose absorption inhibitory

activity of matitol under *in vivo* condition (**experiment 2**) compared to *ex vivo* condition (**experiment 1**) may be due to the disaccharide nature of this particular sugar alcohol. Due to its disaccharide nature, maltitol is hydrolysable by intestinal disaccharidases under *in vivo* condition (Kamoi 1975; Rennhard & Bianchine 1975; Lian-Loh *et al*., 1982), which may also affect its effects *in vivo*. These findings also seem to provide further explanations for the apparent discrepancy on the previously observed inhibitory effects of maltitol on alpha glucosidase and alpha amylase activities *in vitro* (Kang *et al*., 2014), which was not mimicked under *in vivo* experimental condition (Matsuo 2003). Based on the above discussions, it appears that maltitol may not be a useful dietary supplement to ameliorate hyperglycemia via reducing intestinal glucose absorption or enhancing muscle glucose uptake in either normal or diabetic condition. However, it may be useful for glycemic control compared to sucrose, due to its lower glycemic and caloric values.

Myo-inositol, a six-carbon cyclic sugar alcohol was the second sugar alcohol under study. This sugar alcohol exhibited a notable inhibitory effect on intestinal glucose absorption under *ex vivo* condition (**Table 7.1**). The inhibitory activity of myo-inositol on intestinal glucose absorption was further confirmed *in vivo* (**experiment 2**), when dietary myo-inositol (1 g/kg bw) inhibited glucose absorption in the 1st qtr of the small intestine of normal and diabetic animals. This inhibitory activity of myo-inositol impaired postprandial blood glucose increase and was also comparable to that of acarbose, a known anti-diabetic drug. Myo-inositol also delayed gastric emptying in diabetic animals, which may also contribute to its inhibitory activity on intestinal glucose absorption (Salminen *et al*., 1984; Shafer *et al*., 1987). These data suggest that myo-inositol may be useful in the management of postprandial glycemia via inhibiting intestinal glucose absorption. On the other hand, the promising muscle glucose uptake effect of myo-inositol observed in our *ex-vivo* study (**experiment 1**) (**Table 7.1**) again supports the involvement of myo-inositol in the improvement of glucose metabolism via insulin signaling (Dang *et al*., 2010; Santamaria *et al*., 2012), but still requires further investigations. Thus, dietary myo-inositol may be further investigated as an anti-hyperglycaemic supplement for diabetic foods and food products that may be useful in managing hyperglycemia possibly via inhibiting intestinal glucose absorption and enhancing muscle glucose uptake.

The next sugar alcohol under study was sorbitol, which also showed promising *ex vivo* inhibitory effect on intestinal glucose absorption that was even more remarkable than that of myo-inositol (**Table 7.1**). Although the inhibitory effect of sorbitol on intestinal glucose absorption has been previously hypothesized (WHO, 1987), our *in vivo* study (**experiment 2**) was able to confirm this hypothesis and also supports similar inhibitory activity of sorbitol observed in our *ex vivo* study (**experiment 1**). In fact, dietary sorbitol (0.4 g/kg bw) did not only inhibit glucose absorption in the first quarter of the small intestine of normal and diabetic animals, it also delayed gastric emptying and accelerated digesta transit, especially in diabetic animals, which possibly contributed to its inhibitory activity on intestinal glucose absorption (Salminen *et al*., 1984; Shafer *et al*., 1987). Like myo-inositol, the intestinal glucose absorption inhibitory effect of sorbitol was also comparable to acarbose, thus reducing posprandial glucose increase in diabetic animals. These data suggest that sorbitol may be useful in the management of postprandial hyperglycemia, especially in diabetics. On the other hand, our *ex vivo* study (**experiment 1**) is the first study to demonstrate the muscle glucose uptake potentials of sorbitol, which may be further investigated as a possible anti-hyperglycemic mode of action of sorbitol.

At this point, it is important to note that although our *in vivo* absorption study (**experiment 2**) showed that a bolus dose of myo-inositol and sorbitol impaired postprandial blood glucose increase in diabetic animals, it could not be confirmed from our study whether the inhibition of intestinal glucose absorption was solely responsible for this effect or not. It could be hypothesized that myo-inositol and sorbitol also influenced blood glucose increase by enhancing muscle glucose uptake as seen in our *ex vivo* study with both sugar alcohols. However, this hypothesis can only be confirmed following further in depth studies on both sugar alcohols, since our muscle glucose uptake study was limited to an *ex vivo* condition only, with respect to myo-inositol and sorbitol.

The last sugar alcohol under this study was erythritol, a four carbon sugar alcohol. This sugar alcohol is unique because it is the only sugar alcohol that is not metabolized in the body, thus has no glycemic and caloric effect (livesey, 2003). Although this sugar alcohol showed no significant inhibitory effect on intestinal glucose absorption *ex vivo* (experiment 1) (**Table 7.1**), it (1 g/kg bw erythritol) surprisingly inhibited ($p < 0.05$) glucose absorption in the 1st quarter of the small intestine and concomitantly delayed ($p < 0.05$) gastric emptying in both normal

diabetic animals (**experiment 2**). Considering the trend of these data (from *ex vivo* to *in vivo*), we can suggest that the delayed gastric emptying might be a major influencing factor for the intestinal glucose absorption inhibitory effect of erythritol *in vivo* (Salminen*et al*., 1984; Shafer *et al*., 1987). On the other hand our *ex vivo* study (**experiment 1**) also revealed that erythritol exhibited the highest muscle glucose uptake activity among all sugar alcohols tested with or without insulin (**Table 7.1**), which suggests that erythritol might improve muscle glucose uptake via improving some factors in insulin signaling pathway. In order to verify this, erythritol was subjected to an *in vivo* muscle glucose uptake study (**experiment 3**). Data from this study revealed that a bolus dose of erythritol appreciably improved glucose tolerance, insulin secretion, mRNA expression of muscle Glut-4 and IRS-1 as well as the activities of muscle and liver hexokinase, but suppressed the hepatic release of glucose in diabetic animals. These results support the data of our *ex vivo* glucose uptake study (**experiment 1**) on erythriol and also suggest the potency of erythritol to improve glucose tolerance and metabolism via improving insulin secretion and modulating insulin-mediated muscle glucose uptake, thus ameliorate hyperglycemia. Although the inhibitory (**experiment 2**) and glucose uptake (**experiment 3**) effects of erythritol were observed separately in two separate *in vivo* studies, we propose that this bi-functional anti-hyperglycemic potency of erythritol (inhibition of small intestinal glucose absorption and enhancement insulin-mediated muscle glucose uptake and metabolism) may work in concert to improve glucose tolerance and ameliorate hyperglycemia, especially in diabetic animals. Hence, erythritol may be a useful dietary supplement for managing hyperglycemia in diabetic individuals, especially type 2.

7.2   General conclusions

In summary, four sugar alcohols were investigated in this study. Three of these sugar alcohols (myo-inositol, sorbitol and erythritol) demonstrated remarkable inhibitory effects on intestinal glucose absorption in normal and diabetic rats and were also able to appreciably enhance muscle glucose uptake *ex vivo* (**Fig. 7.1** and **7.2**). Erythritol had the most potent muscle glucose uptake activity, which was further confirmed *in vivo* as a possible modulator of insulin-mediated muscle glucose uptake, especially in diabetics (**Fig. 7.2**). This study has identified possible mode of actions through which some commonly used sugar alcohols can exert anti-hyperglycemic effect

in diabetic conditions (**Fig. 7.1** and **7.2**). Thus, these sugar alcohols may be further investigated as potential anti-hyperglycemic supplements that may be useful not only as sweeteners but also in the management of diabetes, especially type 2.
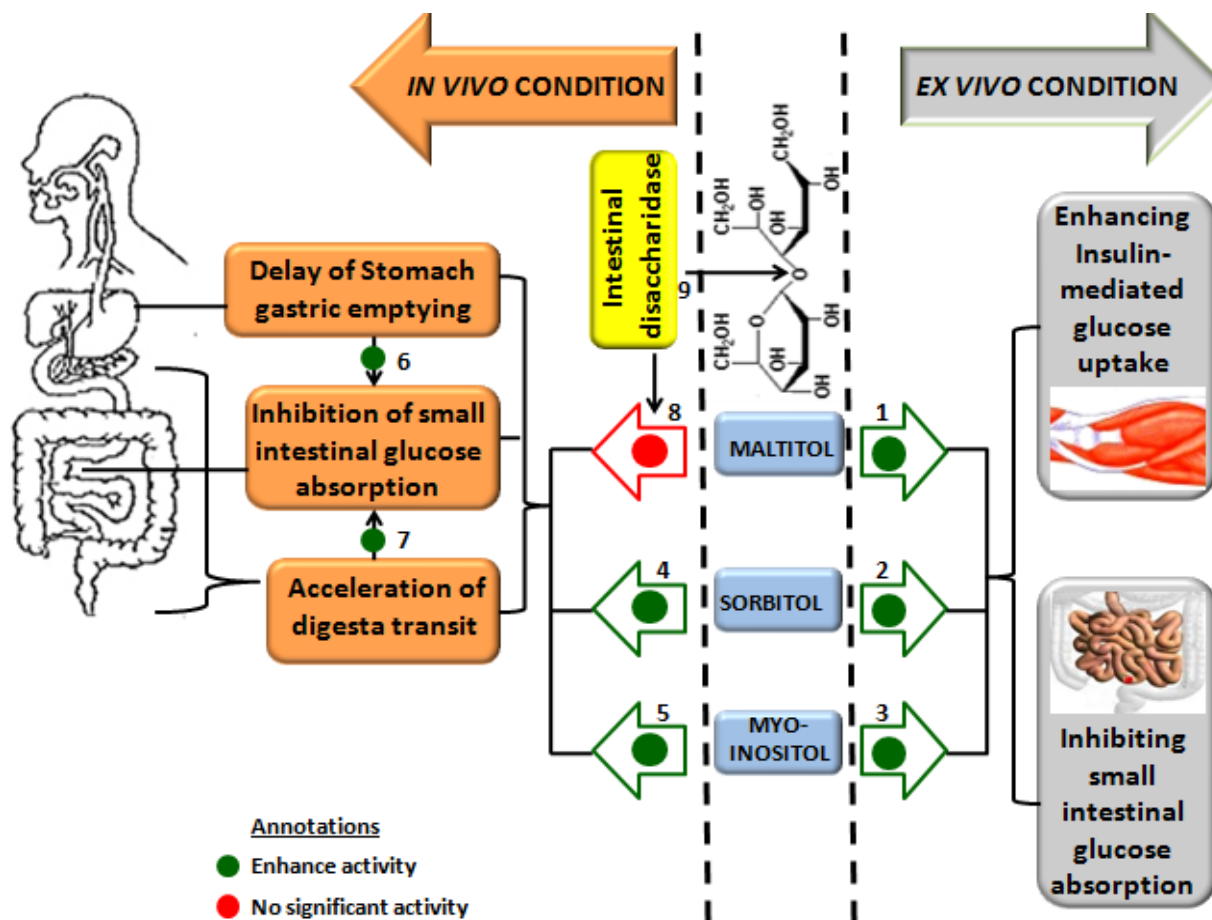

Figure 7.1: Schematic diagram showing the possible anti-hyperglycemic mechanisms of action of sorbitol, myo-inositol and maltitol. Sorbitol myo-inositol, and matitol inhibits intestinal glucose absorption and enhances insulin-mediated muscle glucose uptake *ex vivo* (**1, 2 & 3**). Sorbitol and myo-inositol reduced intestinal glucose absorption *in vivo* (**4 & 5**), which may partly be due to delaying gastric emptying (**6**) and accelerating degista transit (**7**). Maltitol did not significantly influence intestinal glucose absorption and gastric emptying *in vivo* (**8**), possible due to its hydrolysis by intestinal disaccharidases (**9**).
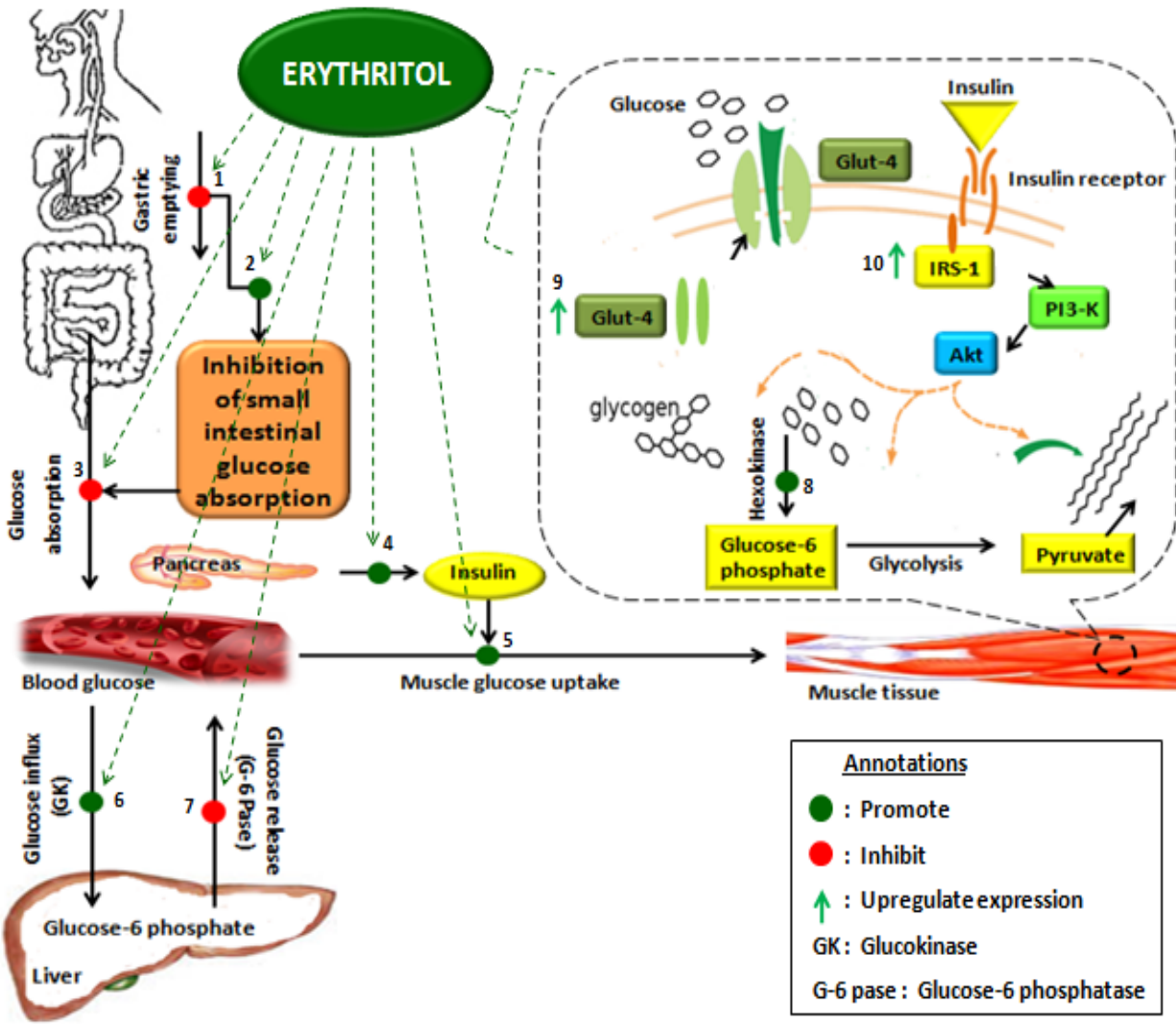
Figure 7.2: Schematic diagram showing the possible anti-hyperglycemic mechanisms of action of erythritol. Erythritol inhibited intestinal glucose absorption (**3**), possibly via delaying gastric emptying (**1 & 2**). Erythritol also enhanced insulin-mediated glucose uptake (**5**), possibly via improving insulin secretion (**4**), mRNA expression of muscle Glut-4 (**9**) and IRS-1 (**10**) as well as the activities of muscle hexokinase (**8**) and liver glucokinase (**6**), while suppressing the hepatic glucose release or glucose-6 phosphatase activity (**7**).

7.3   Recommendations

We recommend that more resources should be channeled into thorough investigations on the potential usefulness of most of these sugar alcohols, specifically for their possible clinical

relevance in the management of diabetes, especially type 2. Considering the affordability and safety concerns of most synthetic anti-diabetic drugs, the use of sugar alcohol may be a safer option to improve the therapeutic value and effectiveness of already existing therapies for the management of type 2 diabetes.

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APPENDIX 1

PUBLISHED ARTICLES FROM THIS THESIS

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ORIGINAL PAPER

Myo-inositol inhibits intestinal glucose absorption and promotes muscle glucose uptake: a dual approach study

Chika Ifeanyi Chukwuma · Mohammed Auwal Ibrahim · Md. Shahidul Islam

Abstract The present study investigated the effects of myo-inositol on muscle glucose uptake and intestinal glucose absorption ex vivo as well as in normal and type 2 diabetes model of rats. In ex vivo study, both intestinal glucose absorption and muscle glucose uptake were studied in isolated rat jejunum and psoas muscle respectively in the presence of increasing concentrations (2.5 % to 20 %) of myo-inositol. In the in vivo study, the effect of a single bolus dose (1 g/kg bw) of oral myo-inositol on intestinal glucose absorption, blood glucose, gastric emptying and digesta transit was investigated in normal and type 2 diabetic rats after 1 h of co-administration with 2 g/kg bw glucose, when phenol red was used as a recovery marker. Myo-inositol inhibited intestinal glucose absorption (IC_{50} = 28.23 ± 6.01 %) and increased muscle glucose uptake, with (GU_{50} = 2.68 ± 0.75 %) or without (GU_{50} = 8.61 ± 0.55 %) insulin. Additionally, oral myo-inositol not only inhibited duodenal glucose absorption and reduced blood glucose increase, but also delayed gastric emptying and accelerated digesta transit in both normal and diabetic animals. Results of this study suggest that dietary myo-inositol inhibits intestinal glucose absorption both in ex vivo and in normal or diabetic rats and also promotes muscle glucose uptake in ex vivo condition. Hence, myo-inositol may be further investigated as a possible anti-hyperglycaemic dietary supplement for diabetic foods and food products.

Keywords Myo-inositol · Type 2 diabetes · Intestinal glucose absorption · Muscle glucose uptake · Rats

Abbreviations

DBC	Diabetic control
DMI	Diabetic myo-inositol
GAI	Glucose absorption index
GIT	Gastrointestinal tract
NC	Normal control
NMI	Normal myo-inositol
PCOS	Polycystic ovary syndrome
PR	Phenol red
T2D	Type 2 diabetes

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Introduction

Recent data indicate that about 415 million people have diabetes worldwide and this figure is likely to increase to about 642 million by 2040 [16]. Among two major types of diabetes (type 1 and type 2), type 2 is the most prevalent one, accounting for more than 90 % of all diabetic cases [20]. Type 2 diabetes (T2D) is a heterogeneous disorder characterized by insulin resistance followed by partial pancreatic β-cell dysfunction [28], leading to persistent hyperglycaemia. The chronic

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RESEARCH ARTICLE

Maltitol inhibits small intestinal glucose absorption and increases insulin mediated muscle glucose uptake *ex vivo* but not in normal and type 2 diabetic rats

Chika Ifeanyi Chukwuma[a], Mohammed Auwal Ibrahim[a,b] and Md. Shahidul Islam[a]

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[b]Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria

ABSTRACT

This study investigated the effects of maltitol on intestinal glucose absorption and muscle glucose uptake using *ex vivo* and *in vivo* experimental models. The *ex vivo* experiment was conducted in isolated jejunum and psoas muscle from normal rats. The *in vivo* study investigated the effects of a single bolus dose of maltitol on gastric emptying, intestinal glucose absorption and digesta transit in normal and type 2 diabetic rats. Maltitol inhibited glucose absorption in isolated rat jejunum and increased glucose uptake in isolated rat psoas muscle in the presence of insulin but not in the absence of insulin. In contrast, maltitol did not significantly ($p > 0.05$) alter small intestinal glucose absorption or blood glucose levels as well as gastric emptying and digesta transit in normal or type 2 diabetic rats. The results suggest that maltitol may not be a suitable dietary supplement for anti-diabetic food and food products to improve glycemic control.

Introduction

Global estimation of diabetes mellitus has revealed that approximately 415 million people were affected with the disease in 2015, and by 2040, this figure would become ~642 million (International Diabetes Federation 2015). Type 2 diabetes (T2D) is the most common form of diabetes mellitus. It is characterized by insulin resistance and pancreatic beta-cell failure, which leads to decrease glucose transport into the muscle and fat cells as well as increase gluconeogenesis and endogenous glucose production in the liver, causing hyperglycemia (Olokoba et al. 2012). T2D is a result of complex interaction between several factors such as genetic, environmental and behavioral risk factors (Olokoba et al. 2012; Wu et al. 2014). However, the most influential factors are the behavioral risk factors such as sedentary life style, consumption of high fat and high calorie diets as well as refined and simple carbohydrates like sucrose and fructose among others (Thorburn et al. 1989; Elliott et al. 2002; Astrup et al. 2002; Hu 2003; Stanhope et al. 2011). Thorburn et al. (1989) reported that normal rats that consumed diet containing 35% of energy as fructose displayed reduced glucose disposal as well as reduced action of insulin in

the liver and most peripheral tissue. Additionally, Stanhope et al. (2011) reported that postprandial triglycerides, LDL-cholesterol, and apolipoprotein-B in young male and female adults were markedly increased after a 12-d consumption of fructose and high fructose corn syrup that was equivalent to 25% of total dietary energy. Furthermore, in human subjects, 10 weeks consumption of sucrose equivalent to 28% of dietary energy increased food intake, body weight, body fat mass, and blood pressure compared to non-sucrose sweetener (Astrup et al. 2002). Hence, there has been a growing acceptance of the use of sugar substitutes as alternative sweetening agents in both non-diabetic and diabetic diets.

Sugar alcohol is one of the classes of sugar substitute that have gained popularity, especially for overweight, obese and diabetic individuals due to their lower caloric value and glycemic response compared to glucose and sucrose as well as minimal or no side effects (Livesey 2003, 2012; Islam & Indarjit 2012; Academy of Nutrition and Dietetics 2012). Maltitol is one of the few commonly used disaccharide sugar alcohol formed from the catalytic hydrogenation of maltose to produce an alpha-1,4 glucose-sorbitol linked

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ARTICLE

Sorbitol increases muscle glucose uptake ex vivo and inhibits intestinal glucose absorption ex vivo and in normal and type 2 diabetic rats

Chika Ifeanyi Chukwuma and Md. Shahidul Islam

Abstract: Previous studies have suggested that sorbitol, a known polyol sweetener, possesses glycemic control potentials. However, the effect of sorbitol on intestinal glucose absorption and muscle glucose uptake still remains elusive. The present study investigated the effects of sorbitol on intestinal glucose absorption and muscle glucose uptake as possible anti-hyperglycemic or glycemic control potentials using ex vivo and in vivo experimental models. Sorbitol (2.5% to 20%) inhibited glucose absorption in isolated rat jejuna ($IC_{50} = 14.6\% \pm 4.6\%$) and increased glucose uptake in isolated rat psoas muscle with ($GU_{50} = 3.5\% \pm 1.6\%$) or without insulin ($GU_{50} = 7.0\% \pm 0.5\%$) in a concentration-dependent manner. Furthermore, sorbitol significantly delayed gastric emptying, accelerated digesta transit, inhibited intestinal glucose absorption, and reduced blood glucose increase in both normoglycemic and type 2 diabetic rats after 1 h of coingestion with glucose. Data of this study suggest that sorbitol exhibited anti-hyperglycemic potentials, possibly via increasing muscle glucose uptake ex vivo and reducing intestinal glucose absorption in normal and type 2 diabetic rats. Hence, sorbitol may be further investigated as a possible anti-hyperglycemic sweetener.

Key words: sorbitol, intestinal glucose absorption, muscle glucose uptake, gastric emptying, type 2 diabetes, rats.

Résumé : D'après des études antérieures, le sorbitol est un polyol bien connu au pouvoir sucrant et ayant un potentiel de contrôle de la glycémie. Toutefois, l'effet du sorbitol sur l'absorption intestinale du glucose et la captation musculaire du glucose n'est pas clair. La présente étude examine les effets du sorbitol sur l'absorption intestinale du glucose et la captation musculaire du glucose sous l'angle du potentiel anti-hyperglycémique ou du contrôle de la glycémie en utilisant des modèles expérimentaux ex vivo et in vivo. Le sorbitol (de 2,5 % à 20 %) inhibe l'absorption du glucose dans le jéjunum isolé du rat ($IC_{50} = 14,6 \pm 4,6$ %) et augmente la captation du glucose dans le muscle psoas isolé du rat avec ($GU_{50} = 3,5 \pm 1,6$ %) ou sans insuline ($GU_{50} = 7,0 \pm 0,5$ %) de manière dépendante de la concentration. De plus, le sorbitol retarde significativement la vidange gastrique, accélère le transit des digesta, inhibe l'absorption intestinale du glucose et atténue l'augmentation de la glycémie chez des rats normoglycémiques et des rats présentant un diabète de type 2 une heure après la consommation du sorbitol avec du glucose. Ces résultats suggèrent que le sorbitol présente un potentiel anti-hyperglycémique possiblement par l'augmentation de la captation musculaire du glucose *ex vivo* et la diminution de l'absorption intestinale du glucose chez des rats normaux et des rats présentant un diabète de type 2. En conséquence, on devrait pousser l'analyse du sorbitol en tant qu'édulcorant anti-hyperglycémique potentiel. [Traduit par la Rédaction]

Mots-clés : sorbitol, absorption intestinale du glucose, captation musculaire du glucose, vidange gastrique, diabète de type 2, rats.

Introduction

Diabetes is one of the major global public health problems and its prevalence is rapidly increasing in all over the world, particularly in the developing nations. According to the International Diabetes Federation, about 415 million people have diabetes worldwide and this figure is likely to become 642 million by 2040 (International Diabetes Federation 2015). Among 2 major types of diabetes, type 2 diabetes (T2D) is the most prevalent one, and accounts more than 90% of all diabetic cases (Loghmani 2005). T2D is a heterogeneous disorder characterized by insulin resistance followed by partial pancreatic β-cell dysfunction (Partley 2013). It is strongly influenced by sedentary life style, high fat and high calorie diet intake as well as routine consumption of refined and simple carbohydrates, e.g., sucrose, fructose, and so on (Uusitupa 2002; Hu 2003; Montonen et al. 2007; Stanhope et al. 2011).

Excessive dietary intake of sucrose- and fructose-containing foods and food products has been consistently associated with detrimental health outcomes, such as elevated food intake, body weight gain, visceral adiposity, reduced insulin sensitivity, lipid dysregulation, and the risk of developing T2D (Thorburn et al. 1989; Astrup et al. 2002; Elliott et al. 2002; Stanhope et al. 2011). Therefore, there has been an upsurge on the use of alternative sweetening agents, such as sugar alcohols.

Sugar alcohol is one of the classes of sugar substitute that have gained great popularity, especially for overweight, obese, and diabetic individuals because of their sweetening ability with lesser undesired calories compared with sucrose (Islam and Indrajit 2012). Additionally, apart from their minimal effect on the integrated blood glucose level compared with sucrose (Talbot and Fisher 1978; Livesey 2003), most commonly used sugar alcohols have been demonstrated to be useful in oral health care (Mäkinen 2010; Gupta et al. 2013). Sorbitol is one of the widely used sugar alcohols. Several studies have reported that sorbitol possesses glycemic control effects, but may also possess anti-hyperglycemic effects

Xylitol: One Name, Numerous Benefits

Chika Ifeanyi Chukwuma and Md. Shahidul Islam

Contents

Abstract

Xylitol among other sugar alcohols have been extensively studied, showing numerous beneficial effects and potential clinical uses other than being used as a sweetener. The present chapter focuses on the numerous beneficial effects of xylitol and its potential clinical relevance. It also elaborated the several beneficial effects of xylitol that requires more investigation, especially at clinical levels to ascertain its clinical therapeutic applications. Information from different sources, majorly from "PubMed" journals, were reviewed, focusing on the beneficial

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APPENDIX 2

SUBMITTED ARTICLES FROM THIS THESIS

Subject:	Re: Molecular Nutrition and Food Research - Decision on Manuscript # mnfr.201601105
From:	Chika Chukwuma (chykochi@yahoo.com)
To:	sislam1974@yahoo.com;
Date:	Friday, 20 January 2017, 6:47

Dear Prof Islam
Thank you for the message will see you this morning.

Regards,
Chika

Sent from Yahoo Mail on Android

On Thu, Jan 19, 2017 at 10:16, Md. Shahidul Islam
<sislam1974@yahoo.com> wrote:

> Dear Chika:
> Please find below the review report on your Erythritol article. We need to sit together urgently not only to complete the revision but also we may need to do some additional works particularly for protein expression. Please see me as soon as possible.
>
> Regards,
> Prof. Islam
> --
>
>
>
> On Thursday, January 19, 2017 8:44 AM, Molecular Nutrition and Food Research <onbehalfof+mnf+wiley.com@manuscriptcentral.com> wrote:
>
>
> 19-Jan-2017
>
> Dear Dr. Islam:
>
> Manuscript # mnfr.201601105 entitled "Erythritol reduces small intestinal glucose absorption, increases muscle glucose uptake, improves glucose metabolic enzymes activities and increases mRNA expression of Glut-4 and IRS-1 in type 2 diabetic rats" which you submitted to Molecular Nutrition & Food Research has been reviewed. The comments of the referees are included at the end of this email.
>
> A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication.
> Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision may be subject to re-review by the referees before a decision is rendered.

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APPENDIX 3

OTHER PUBLISHED AND SUBMITTED ARTICLES

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Flowers of *Clerodendrum volubile* exacerbate immunomodulation by suppressing phagocytic oxidative burst and modulation of COX-2 activity

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A R T I C L E I N F O

A B S T R A C T

The immunomodulatory potentials of the crude methanolic extract and fractions [n-hexane (Hex), n-dichloromethane (DCM), ethyl acetate (EtOAc) and n-butanol (BuOH)] of *Clerodendrum volubile* flowers were investigated on whole blood phagocytic oxidative burst using luminol-amplified chemilumines-cence technique. They were also investigated for their free radicals scavenging activities. The DCM fraction showed significant ($p < 0.05$) anti-oxidative burst and free radical scavenging activities indicating high immunomodulatory and antioxidant potencies respectively. Cytotoxicity assay of the DCM fraction revealed a cytotoxic effect on CC-1 normal cell line. GCMS analysis revealed the presence of triacetin; 3,6-dimethyl-3-octanol; 2R – Acetoxymethyl-1,3,3-trimethtyl – 4 t – (3-methyl-2-buten-1-yl) – 1c – cyclohexanol and Stigmastan – 3,5–diene in DCM fraction. These compounds were docked with the active sites of cyclooxygenase-2 (COX-2). Triacetin, 3,6-dimethyl-3-Octanol, and 2R-Acetoxymethyl-1,3,3-trimethtyl-4t-(3-methyl-2-buten-1-yl)-1c-cyclohexanol docked comfortably with COX-2 with good scoring function (-CDocker energy) indicating their inhibitory potency against COX-2. 3,6–dimethyl-3-Octanol, displayed the lowest predicted free energy of binding ($-21.4 \text{ kcal mol}^{-1}$) suggesting its stronger interaction with COX-2, this was followed by 2R – Acetoxymethyl-1, 3, 3-trimethtyl-4t-(3-methyl-2-buten-1-yl)-1c-cyclhexanol ($BE = -20.5 \text{ kcal mol}^{-1}$), and triacetin ($BE = -10.9 \text{ kcal mol}^{-1}$). Stigmastan – 3,5–diene failed to dock with COX-2. The observed suppressive effect of the DCM fraction of *C. volubile* flower methanolic extract on phagocytic oxidative burst indicates an immunomodulatory potential. This is further reflected in its free scavenging activities and synergetic modulation of COX-2 activities by its identified compounds *in silico*.

1. Introduction

Reactive oxygen species (ROS) has long been shown to be produced during mitochondrial electron transport chain and/or oxido-reductase and metal–catalyzed oxidative reactions in aerobic tissues [1]. Under normal physiological condition, these free radicals are scavenged by antioxidants. However, oxidative stress sets in when there is a depletion of antioxidants or when the production of free radicals overwhelms the body's antioxidant system causing an imbalance between the free radicals and the antioxidant system [2]. Oxidative stress has been implicated in the peroxidation of membrane lipids, oxidation of protein sulfhydryl groups and disruption of the DNA [1].

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Effects of xylitol on carbohydrate digesting enzymes activity, intestinal glucose absorption and muscle glucose uptake: a multi-mode study

Chika Ifeanyi Chukwuma and Md. Shahidul Islam*

The present study investigated the possible mechanism(s) behind the effects of xylitol on carbohydrate digesting enzymes activity, muscle glucose uptake and intestinal glucose absorption using *in vitro*, *ex vivo* and *in vivo* experimental models. The effects of increasing concentrations of xylitol (2.5%–40% or 164.31 mM–2628.99 mM) on alpha amylase and alpha glucosidase activity *in vitro* and intestinal glucose absorption and muscle glucose uptake were investigated under *ex vivo* conditions. Additionally, the effects of an oral bolus dose of xylitol (1 g per kg BW) on gastric emptying and intestinal glucose absorption and digesta transit in the different segments of the intestinal tract were investigated in normal and type 2 diabetic rats at 1 hour after dose administration, when phenol red was used as a recovery marker. Xylitol exhibited concentration-dependent inhibition of alpha amylase (IC_{50} = 1364.04 mM) and alpha glucosidase (IC_{50} = 1127.52 mM) activity *in vitro* and small intestinal glucose absorption under *ex vivo* condition. Xylitol also increased dose dependent muscle glucose uptake with and without insulin, although the uptake was not significantly affected by the addition of insulin. Oral single bolus dose of xylitol significantly delayed gastric emptying, inhibited intestinal glucose absorption but increased the intestinal digesta transit rate in both normal and diabetic rats compared to their respective controls. The data of this study suggest that xylitol reduces intestinal glucose absorption *via* inhibiting major carbohydrate digesting enzymes, slowing gastric emptying and fastening the intestinal transit rate, but increases muscle glucose uptake in normal and type 2 diabetic rats.

1. Introduction

Starch from carbohydrates is a major dietary source of glucose, which is produced by the gastrointestinal hydrolysis of starch by α-amylase and α-glucosidase enzymes.[1] Then it is absorbed *via* small intestinal mucosa and influences postprandial blood glucose levels and hyperglycemic condition in diabetics.[1] Thus, limiting the extent of postprandial glucose production as well as absorption can significantly suppress hyperglycemia as well as other complications in diabetics. This is because persistent hyperglycemia has been reported as a major culprit for diabetes associated complications in all forms of diabetes, with type 2 diabetes (T2D) having the highest prevalence.[2] About 90–95% of the total diabetic patients are suffering from T2D, which has been defined as a heterogeneous metabolic disorder caused by insulin resistance followed by partial pancreatic beta-cell dysfunction as well as hyperglycemia.[2–4]

Recently, there has been a growing interest in using nutraceuticals for the management of hyperglycemia as well as T2D,[5] which includes but not limited to medicinal foods, functional foods and sugar alcohols such as xylitol.

Xylitol is a 5 carbon sugar alcohol with a lower glycemic index (13 *vs.* 65) and calorific value (2.4 *vs.* 4.0 kcal g^{-1}) compared to sucrose.[6,7] A number of previous studies reported that xylitol has many other potential beneficial effects such as the control and prevention of obesity, diabetes and related metabolic disorders.[8] In a recent study, Islam[6] reported that 3 weeks supplementation of 10% dietary xylitol significantly decreased non-fasting blood glucose (NFBG) and serum fructosamine levels; increased the serum insulin levels; and improved the glucose tolerance ability compared to 10% sucrose in non-diabetic rats.[6] In a more recent study, Islam and Indarjit[9] reported that 5 weeks supplementation of 10% dietary xylitol significantly reduced NFBG and also improved most of the diabetes-related metabolic parameters in a T2D rat model.[9] On the other hand, Amo *et al.*[8] reported that 8-week supplementation of 1 or 2 g of xylitol per 100 kcal diet significantly decreased visceral fat mass and plasma lipid concentration in high fat diet-fed rats.[8] In another study, Kishore

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XYLITOL IMPROVES ANTI-OXIDATIVE DEFENSE SYSTEM IN SERUM, LIVER, HEART, KIDNEY AND PANCREAS OF NORMAL AND TYPE 2 DIABETES MODEL OF RATS

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Running title:

Anti-oxidative effects of xylitol.

Abstract

The present study investigated the anti-oxidative effects of xylitol both *in vitro* and *in vivo* in normal and type 2 diabetes (T2D) rat model. Free radical scavenging and ferric reducing potentials of different concentrations of xylitol were investigated*in vitro*. For *in vivo* study, six weeks old male Sprague-Dawley rats were divided into four groups, namely: Normal Control (NC), Diabetic Control (DBC), Normal Xylitol (NXYL) and Diabetic Xylitol (DXYL). T2D was induced in the DBC and DXYL groups. After the confirmation of diabetes, a 10% xylitol solution was supplied instead of drinking water to NXYL and DXYL, while normal drinking water was supplied NC and DBC *ad libitum*. After five weeks intervention period, the animals were sacrificed and thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH) concentrations as well as superoxide dismutase, catalase glutathione reductase and glutathione peroxidase activities were determined in the liver, heart, kidney, pancreatic tissues and serum samples. Xylitol exhibited significant ($p<0.05$) *in vitro* nitric oxide and hydroxyl radical scavenging and ferric reducing activities. *In vivo* study revealed significant ($p<0.05$) reduction in TBARS concentrations in the xylitol consuming groups compared to their respective controls. Significant ($p<0.05$) increase in GSH levels and antioxidant enzyme activities were observed in analyzed tissues and serum of xylitol-fed animals compared to their respective controls. Results of this study indicate that xylitol has strong anti-oxidative potential against T2D-associated oxidative stress. Hence, xylitol can be used as a potential supplement in diabetic foods and food products.
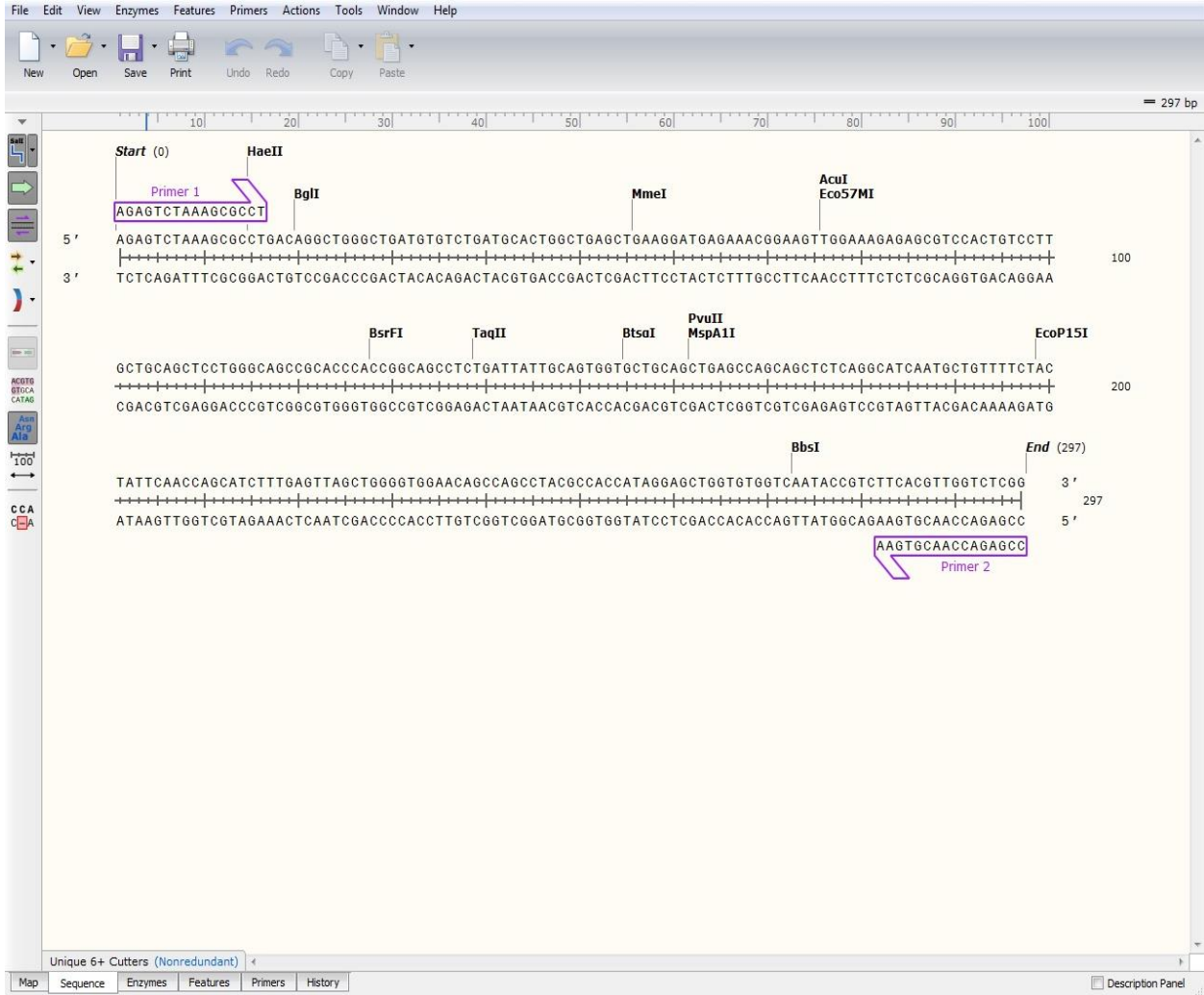
Keywords: Xylitol, Antioxidants, Oxidative stress, Sugar substitute, Type 2 diabetes.
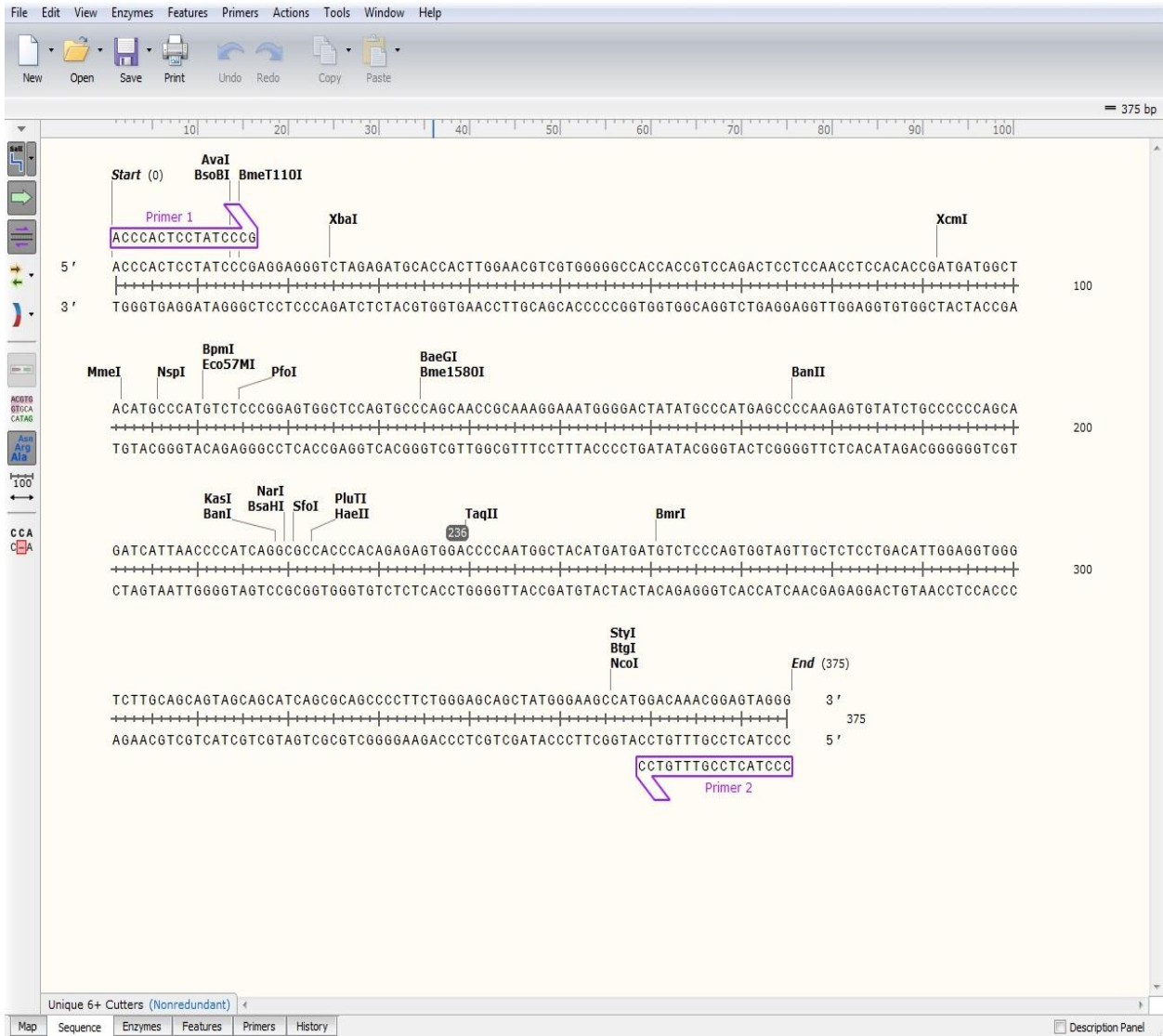
# APPENDIX 4

SUPPLEMENTARY DATA OF THIS THESIS
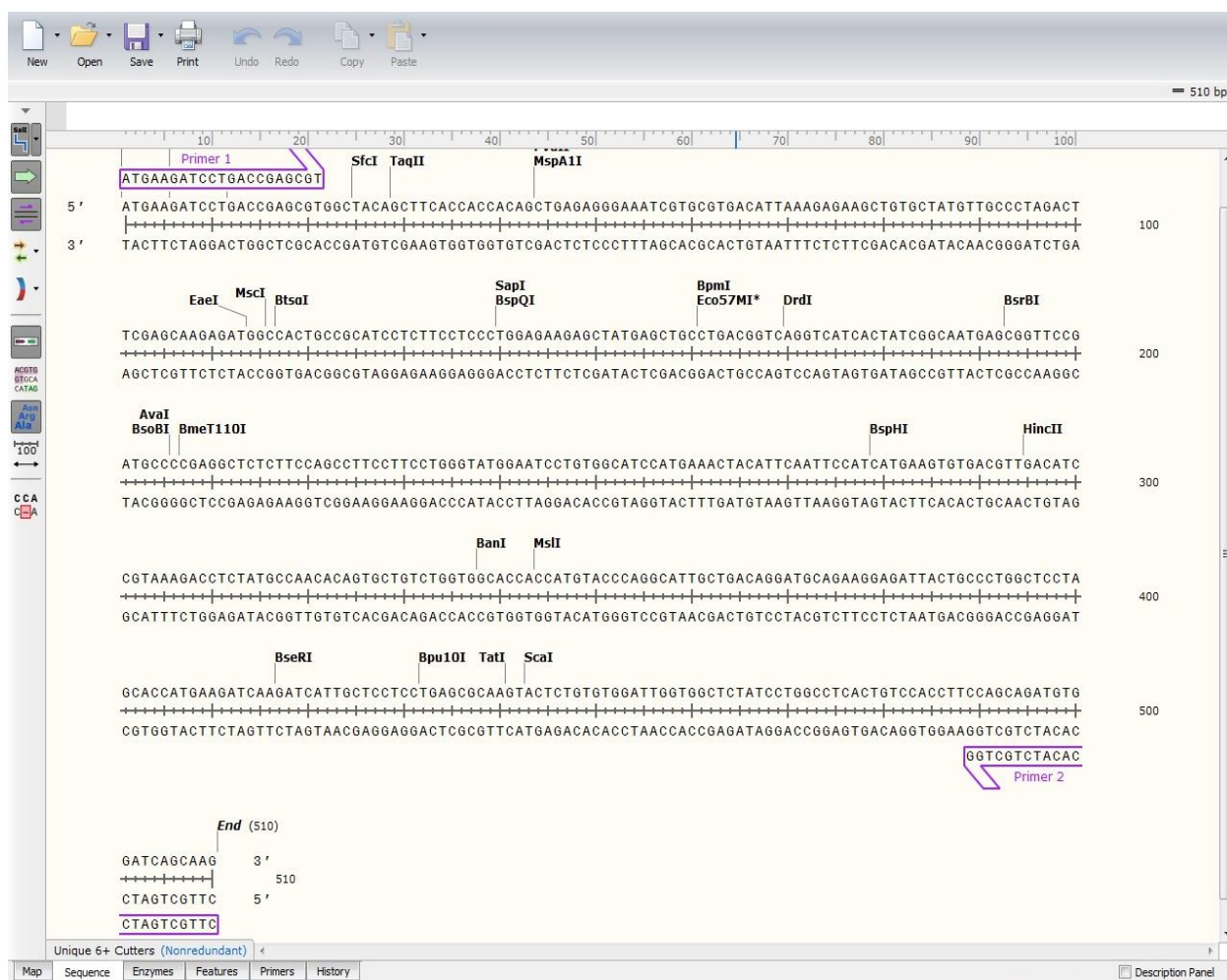
Supplementary data 1: Screen shots images showing the *in slico* determination of primer compatibility and expected qPCR product sizes of β-Actin target gene.

Supplementary data2: Screen shots images showing the *in slico* determination of primer compatibility and expected qPCR product sizes of Glut-4 target gene.

Supplementary data3: Screen shots images showing the *in slico* determination of primer compatibility and expected qPCR product sizes of IRS-1 target gene.
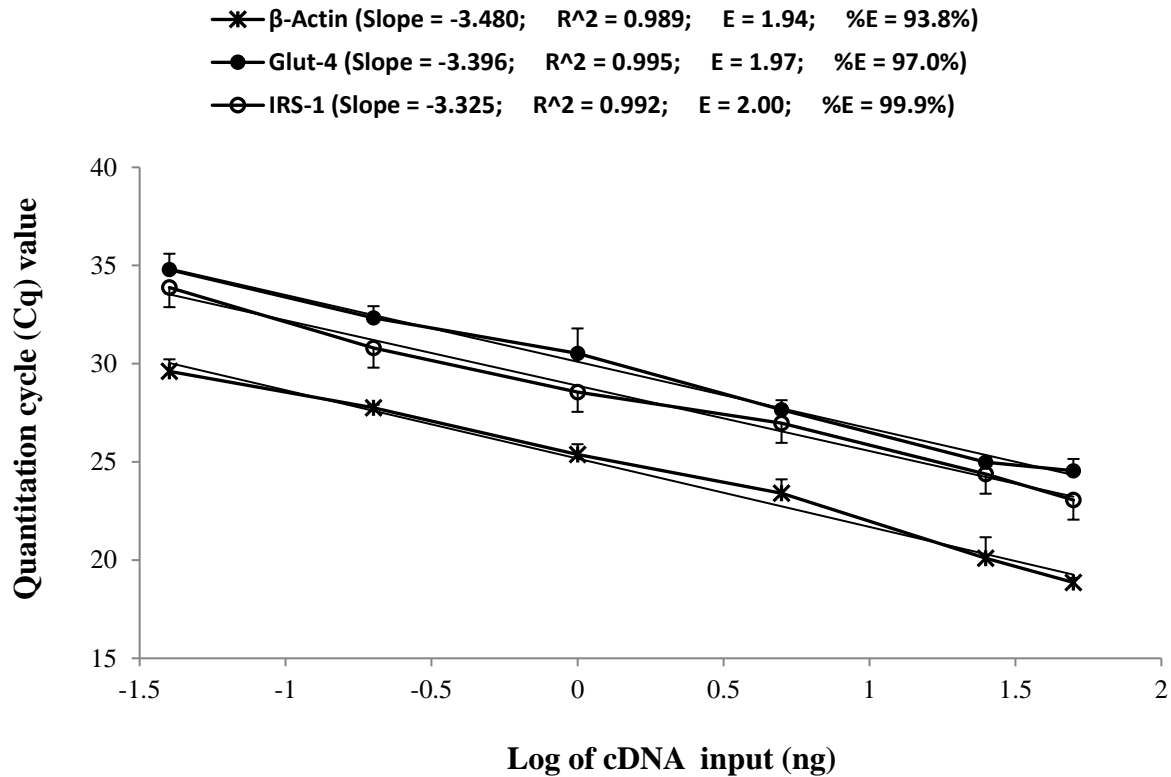
Supplementary data 4: Standard curve for determining qPCR efficiencies of the reference gene (β-Actin) and genes of interest (Glut-4 and IRS-1). Mean Cq values of technical replicate (mean ±SD; n=2) was plotted against the log of the corresponding cDNA input (ng) and the corresponding qPCR efficiencies (E) was calculated using the following formular: $E = 10^{[-1/slope]}$.
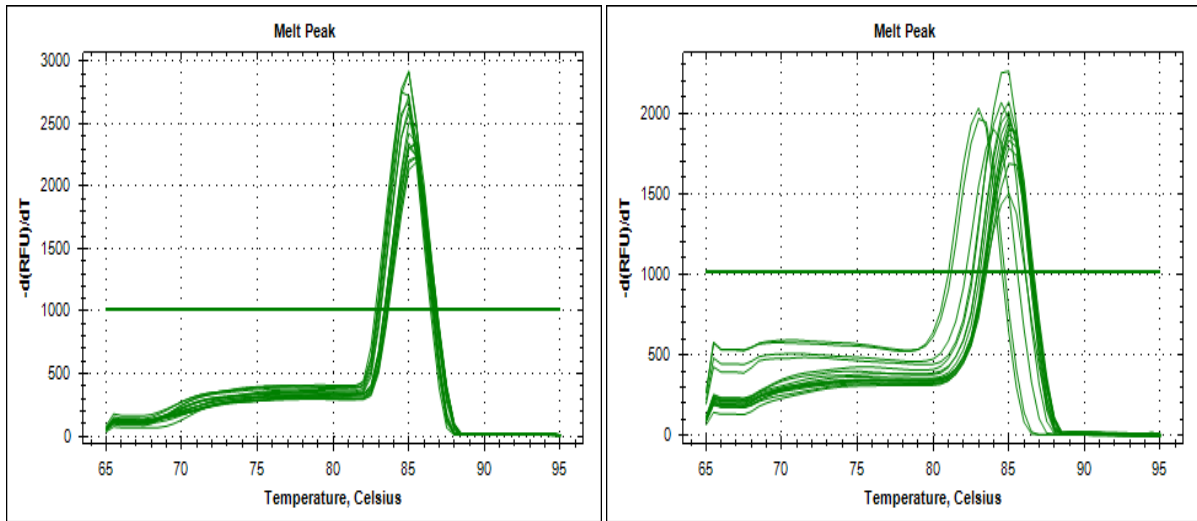
(a)

(b)
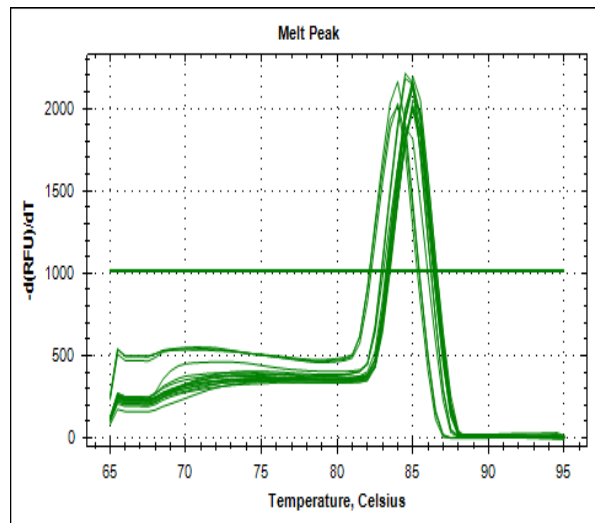
(c)

Supplementary data 5: qPCR melt curves of the different cDNA samples for **(a)** β-Actin, **(b)** Glut-4 and **(c)** IRS-1 target genes.
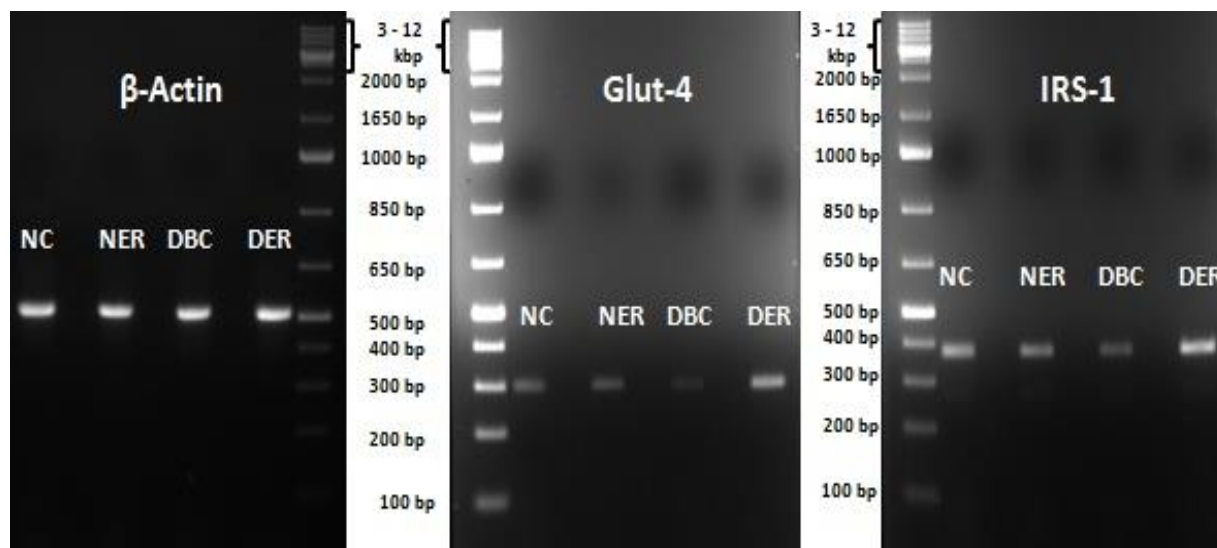
Supplementary data 6: qPCR products of target genes (β-Actin, Glut-4 and IRS-1) subjected to a 2% agarose gel electrophoresis and product sizes estimated with a 1 kb plus ladder. NC, normal control; NER, normal erythritol; DBC, diabetic control; DER, diabetic erythritol.

APPENDIX 5

ETHICAL APPROVAL FOR ANIMAL RESEARCH

Ethical approval 1: The ethical clearance letter obtained from the University Animal Research Ethics Committee to conduct the *ex vivo* (experiment 1) and *in vivo* intestinal glucose absorption study (experiment 2).

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02 December 2015

Mr Chika Ifeanyi Chukwuma (212522184)
School of Life Sciences
Westivlle Campus

Dear Mr Chukwuma,

Protocol reference number: AREC/099/015D
Project title: Studies on the anti-hyperglycemic potentials and possible mode of actions of some commonly used sugar alcohols

Full Approval – Research Application

With regards to your revised application received on 20 October 2015. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 25 August 2016.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

....
Dr Sanil Singh
Deputy Chair: Animal Research Ethics Committee

/ms

Cc Superivsor: Dr S Islam
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Jessica Light
Cc BRU – Dr Linda Bester
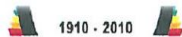

Animal Research Ethics Committee (AREC)
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Ethical approval 2: The ethical clearance letter obtained from the University Animal Research Ethics Committee to conduct the *in vivo* muscle glucose uptake (experiment 3).

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