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SCHOOL OF BIOMEDICAL AND ALLIED HEALTH SCIENCES

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UNIVERSITY OF GHANA, LEGON

ACTION OF NUTRITIVE AND NON-NUTRITIVE SWEETENERS ON INSULIN SIGNALLING AND CARDIOMETABOLIC RISK FACTORS USING AN EXPERIMENTAL ANIMAL MODEL

BY

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DECLARATION

I hereby declare that this thesis is the result of research undertaken by Ruth Tenkoramaa Owu towards the award of the Doctor of Philosophy in Dietetics in the Department of Dietetics, University of Ghana. This was done under the supervision of Prof. George Asare, Dr. Matilda Asante, and Dr. Charles Brown.

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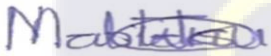

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ABSTRACT

Background: The increase in the production and intake of nutritive sweeteners (NS) has been observed to parallel the rise in cardiometabolic risk factors. Due to health-related effects attributed to the intake of NS, there is an increase in the production and intake of low or no calorie sweeteners (LNCS). However, there is an increasing volume of evidence which implies that LNCS may contribute to the development of metabolic syndrome although they do not contribute to caloric intake. Experimental studies have explored the relationship between the intake of sweeteners (both caloric and non-caloric) on individual components of metabolic syndrome including insulin resistance without examining possible mechanisms involved.

Aim: To determine the effect of nutritive and non-nutritive sweeteners on cardiometabolic risk factors and GLUT4 insulin signalling pathway using an experimental animal model.

Methods: An experimental study was conducted at the Department of Animal Experimentation at the Noguchi Memorial Institute for Medical Research. Thirteen groups of female Sprague-Dawley rats (6 per group) were administered different doses of sweeteners (3 dose groups per sweetener; low, medium and high): white sugar (0.035, 0.07, and 0.1 g/ml), brown sugar (0.036, 0.072, and 0.11 g/ml), honey (0.047, 0.094, and 0.14 g/ml) and stevia (0.007, 0.014, and 0.02 g/ml), control (distilled water) for 17 weeks. A specified amount of food was administered to the rats daily and their daily food consumption was estimated. During the 17 weeks, the rats were weighed weekly and their fasting plasma glucose was measured bi-weekly. At the end of the study, the rats were sacrificed, and their blood samples were obtained by cardiac puncture for further biochemical analysis. Organs (brain, pancreas, and muscles) were harvested for histological analysis. Lipid profiles consisting of total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglyceride (TG), and very low-density lipoprotein (VLDL) were determined at the end of the study. Lipid ratios, Castelli Risk Index I (CRI-I), Castelli Risk Index II (CRI-II), and Atherogenic Index of Plasma (AIP) were

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estimated from the lipid profiles. Serum fasting insulin and serum GLUT4 protein concentrations were determined at the end of the study using a rat ELISA kit. Hexokinase 2 (HK2) enzyme concentration was assessed from muscle homogenate using a rat ELISA kit. The muscles and pancreas were further processed for histological analysis. GraphPad Prism software version 9.0 was used for the statistical analysis. Descriptive variables were expressed as means ± SEM. Statistical significance was set at $p< 0.05$. Two-way mixed ANOVA was used to compare the changes in body weight and fasting plasma glucose over time among the various experimental groups. One-way ANOVA was used to compare differences in the means of different variables (percent weight change, end line fasting plasma glucose, fasting serum, HOMA-IR, TC, TG, LDL, HDL, CRI-I and II and AIP) between the experimental groups. Tukey's post hoc tests were used to detect where differences between the group were observed.

Results: The average weight of all the rats increased over time in all the experimental groups. A two-way ANOVA showed a statistically significant interaction between duration and type of treatment on weight gain [F (28.22, 52.86) = 2.85, $p<0.0001$, $\eta^2 = 0.897$)]. Significant differences in percent weight gain were observed among the various treatment groups [(F (12, 65) = 3.953, $p<0.001$, $\eta^2 = 0.42$)]. Compared to the control, percent weight gain was highest in the experimental group administered with stevia high dose (72.7 ± 10.5) (p = 0.005). There was a statistically significant interaction between the duration of treatment and type of treatment on fasting blood glucose [F (5.116, 199.5) = 56.95, p < 0.001, η^2=0.467]. Fasting Insulin concentration was significantly higher in the control than in all the experimental groups (p<0.0001). Similarly, the HOMA-IR was significantly higher in the control group than in all the treatment groups (p<0.0001). There were significant differences in LDL, HDL, TG, and VLDL between the different treatment groups (p=0.001, 0.038, 0.003, 0.010 respectively). The experimental group administered with high-dose white sugar had the highest CRI-I (1.79 ± 0.11) and CRI-II (0.49 ± 0.09). Both CRI-I and CRI-II had a dose-dependent increase. AIP was

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highest in the high-dose stevia experimental group (0.21 ± 0.07) compared to the control (p = 0.008). The AIP increased in a dose-dependent manner. GLUT4 concentration was highest in the control group (4.38 ± 0.28 ng/ml). There was a significant difference in GLUT4 protein concentration between the different treatment groups [F (12, 45) = 13.09, p<0.0001]. High doses of the sweeteners resulted in a decrease in HK2 within all the sweetener groups. A one-way ANOVA revealed significant differences in HK2 concentration between the different experimental groups [F (12, 64) = 11.66, p=0.0001]. The control rat showed normal islets of Langerhans. The pancreas of high-dose white sugar, high-dose brown sugar, and medium-dose stevia showed some pathological changes. The skeletal muscle of the control rat showed adequately preserved myofibers with clear striation and peripheral myonuclei. Pathological changes were observed in the skeletal muscle of all doses of stevia.

Conclusion: Increased consumption of white sugar and stevia can increase food consumption and weight gain. High intakes of white sugar and stevia may initiate and promote atherosclerosis. Also, the intake of sweeteners in high quantities can affect insulin signalling through a reduction in GLUT4 expression and HK2 which are key factors in the development of type 2 diabetes. Intake of white sugar, brown sugar, and stevia can induce structural changes in the pancreas and skeletal muscle. In comparison with the other sweeteners, honey had a lesser effect on the risk factors of cardiometabolic syndrome.

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DEDICATION

I dedicate this work to God Almighty. To my husband Rev Carroll Kwabena Owu, and my children Joel Owu, Eliana Clara Owu and Ewura Adwoa Owu.

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INTEGRI PROCEDAMUS

University of Ghana http://ugspace.ug.edu.gh

TABLE OF CONTENTS

University of Ghana http://ugspace.ug.edu.gh

University of Ghana http://ugspace.ug.edu.gh

University of Ghana http://ugspace.ug.edu.gh

University of Ghana http://ugspace.ug.edu.gh

University of Ghana http://ugspace.ug.edu.gh

University of Ghana http://ugspace.ug.edu.gh

LIST OF TABLES

University of Ghana http://ugspace.ug.edu.gh

University of Ghana http://ugspace.ug.edu.gh

LIST OF FIGURES

University of Ghana http://ugspace.ug.edu.gh

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LIST OF ABBREVIATIONS

ACK	Acesulfame K
AHA	American Heart Association
AHA/ NHLBI	American Heart Association / National Heart, Lung and Blood Institute
AIP	Atherogenic Index of Plasma
AMPK	Adenosine Monophosphate-activated Protein Kinase
APS	Associated Protein Substrate
ATP	Adenosine Triphosphate
BMI	Body Mass Index
CaMKII	Calcium-calmodulin (CaM)-dependent protein kinase II
CDC	Centre for Disease Control
CHD	Coronary Heart Disease
CMS	Cardio Metabolic Syndrome
COOH	Carboxyl Group
CRI	Coronary Risk Index
CS	Caloric Sweeteners
CVD	Cardiovascular Disease
DAE	Department of Animal Experimentation
DHAP	Dihydroxyacetone Phosphate
EDTA	Ethylenediaminetetraacetic acid
EGIR	European Group for the Study of Insulin Resistance
FBS	Faconi Bekel Syndrome
FDA	Food and Drug Administration
FOS	Framingham Offspring Study
G3P	Glycerol-3-Phosphate

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G6P	Glucose 6 Phosphate
GIP	Gastric Inhibitory Polypeptide
GLP	Glucagon-like-Peptide
GLUT	Glucose Transporter
H&E	Hematoxylosin Eosin
HDL-C	High Density Lipoprotein Cholesterol
HEC	Hyperinsulinemic Euglycemic Pump
HK	Hexokinase
HOMA-IR	Homeostatic Model Assessment – Insulin Resistance
HRP	Horseradish Peroxidase
IAS	International Arteriosclerotic Society
ICCR	International Chair on Cardiometabolic Risk
IDF	International Diabetes Federation
IDL	Intermediate Density Lipoprotein
IR	Insulin Receptor/ Insulin Resistance
JIS	Joint Interim Statement
LDL-C	Low Density Lipoprotein Cholesterol
LNCS	Low or No Calorie Sweeteners
MetS	Metabolic Syndrome
NCEP/ ATP III	National Cholesterol Education Program/ Adult Treatment Plan III
NH_2	Amino Group
NHANES	National Heatlh and Nutrition Examination Survey
NMIMR	Noguchi Memorial Institute for Medical Research
NOS	Nitrous Oxide System
NS	Nutritive Sweeteners

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PDKI	Phosphoinositide Dependent Protein Kinase
PI3K	Phosphatidyl Inositol 3 Kinase
QUICKI	Quantitative Insulin-Sensitivity Check Index
REB	Rebauside
ROS	Reactive Oxygen Species
ROW	Relative Organ Weight
SEM	Standard Error of Mean
SGLT	Sodium Glucose Transporter
SSB	Sugar Sweetened Beverages
TAG	Triacyl glycerol
TC	Total Cholesterol
TG	Triglyceride
TMB	Tetramethylbenzidine
UG	IACUC – University of Ghana Institutional Animal Care and Use Committee
US	United States
USFDA	United States Food and Drug Administration
VLDL	Very Low-Density Lipoprotein
WHO	World Health Organisation

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CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Humans have a natural preference for sweet taste. This has led to an increase in sugar consumption and a desire to eat. As a result, huge amounts of nutritive or caloric and non-nutritive or low and no-calorie sweeteners (LNCS) are being produced (González-Montemayor et al., 2019).

Nutritive sweeteners (NS) or caloric sweeteners (CS) provide energy (~4 kcal/g) (Tou et al., 2011). They contain carbohydrates in the form of simple sugars. Nutritive sweeteners can be naturally present in foods or added to foods during their preparation or manufacturing. The simple sugars present in NS are either monosaccharides or disaccharides (Tou et al., 2011). Monosaccharides (glucose, fructose, and galactose) are the simplified forms of carbohydrates which cannot be broken down further. Disaccharides on the other hand (sucrose, maltose, and lactose) consists of two monosaccharides units (Tou et al., 2011). Disaccharides are further broken down to their individual monosaccharide units. Commonly consumed NS added to food include honey, white granulated sugar, and brown sugar (Das & Chakraborty, 2016). White granulated sugar and brown granulated sugar are produced from sugar cane or sugar beets (Velásquez et al., 2019). Honey on the other hand is an ancient natural sweetener produced by bees from the nectar or secretions of plants (González-Montemayor et al., 2019).

Low and no calorie sweeteners (LNCS) provide minimal or no calories. They are known as "high intensity sweeteners" because of their high sweetener intensities. Small doses are sufficient to be used as replacements for NS. There are currently eight LNCS approved by the United States Food and Drug Administration (FDA) namely: acesulfame-K (ACK), aspartame, neotame, saccharin, sucralose, and stevia, advantame and Luo Han Guo fruit extracts (USFDA,

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2018). The European Union, on the other hand, has recommended 11 LNCS as safe for use; acesulfame-K, aspartame, cyclamate, saccharin, sucralose, thaumatin, neohesperidine, steviol glycosides, neotame, aspartame-acesulfame salt and advantame (Daher et al., 2022). Aside from being used as table-top packets, they are also added to food products like sweetened beverages, bread, cereals, granola bars sugar-free yoghurts or ice-cream, condiments, sugar-free jam, medications (antibiotics, multivitamins, cough syrups), flavoured toothpaste and mouthwash (Sylvetsky & Rother, 2016).

White sugar (sucrose) is a disaccharide composed of two monosaccharide units: glucose and fructose. However, during digestion, the brush border enzymes of the small intestines convert 10-25% of the fructose to glucose and what remains is absorbed into the general circulation (Piggott & Conner, 2003). Brown granulated sugar on the other hand looks similar to white granulated sugar but has a light brown shade due to the presence of molasses (Chen et al., 2021). Brown sugar contains sucrose, glucose, and fructose. Its digestion can be compared to that of white sugar (Piggott & Conner, 2003). Honey, another common nutritive sweetener has carbohydrates (fructose, glucose, sucrose, and maltose) constituting 60 to 85%. It is also made up of water, some minerals and vitamins, proteins, amino acids, phenols, flavonoids and enzymes (glucose oxidase, diastase and invertase) (Tou et al., 2011; Castro-Muñoz et al., 2022).

Glucose and fructose are the main end products in the digestion and metabolism of nutritive sweeteners. Sucrose and maltose found in caloric sweeteners are broken down to glucose and fructose before it is absorbed. Glucose and fructose are absorbed via the glucose transporter GLUT4 and GLUT 5 respectively (Noelting & DiBaise, 2015). The absorbed fructose serves as a precursor to produce fatty acids in the liver. This is a risk factor in the development of metabolic disorders (Nguyen et al., 2019a). Glucose on the other hand is the body's primary

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source of energy. Postprandial concentrations are maintained through GLUT4; an insulin-regulated glucose transporter (Sayem et al., 2018; Bryant & Gould, 2020). Glucose transporter 4 is responsible for the movement of glucose into the adipocytes, skeletal and cardiac muscles (Vargas & Carrillo Sepulveda, 2019). An increase in the glucose level in the blood triggers insulin release from the pancreatic beta cells. When insulin binds to its receptor it triggers the insulin signalling pathway. This causes the exocytosis of a large number of GLUT4 transporter proteins to the surface of the cell membrane and subsequent uptake of glucose by the GLUT4 vesicles into the muscles and adipose tissues (Vargas & Carrillo Sepulveda, 2019; Thorens & Mueckler, 2010). The muscle is responsible for removing 70% of glucose from the general circulation. Glucose uptake is followed by its activation by hexokinase II to glucose-6-phosphate (van Schattingen, 2021). Insulin resistance occurs when insulin sensitive cells in the muscles, fat and liver are not able to respond or ignore insulin signalling affecting their ability to easily take up glucose from the blood. Impaired translocation and or a decrease in GLUT4 transporter protein is observed in metabolic syndrome (MetS) alongside downregulating hexokinase II (Leguisamo et al., 2012a; Pendergrass et al., 1998). Altered insulin signalling results in impaired glucose tolerance which over time develops into type 2 diabetes (T2D) (National Institute of Diabetes and Digestive and Kidney Diseases, 2018).

Low and no calorie sweeteners on the other hand are mostly not absorbed directly into the body but excreted via the kidneys and faeces and metabolized by intestinal microbiota (Ishiguro et al., 2018). Based on their metabolic pathway, they are classified into two (2) groups; the first group undergoes almost no metabolism and minimal absorption (Acesulfame K, saccharin, and sucralose). The second group of LNCS are digested and metabolized in the intestines with their end-products absorbed and metabolised systematically (aspartame, steviol glycosides) (Magnuson et al., 2016). Steviol glycosides are chemical compounds found in the plant *Stevia rebaudiana*. Steviol is hydrolysed by Bacteroides in the colon (Magnuson et al., 2016).

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Negative health implications associated with excessive intake of caloric sweeteners and T2D dates to the 1960 (Tou et al., 2011). The consumption of caloric sweeteners in the dietary management of T2D remains controversial due to their rapid rate of absorption resulting in elevated blood glucose levels. However, in the 1990s there was a shift in focus to caloric sweeteners and hyperactivity syndrome and dental caries in children and adolescents. In the 2000s, the health implications of caloric sweeteners were expanded to include obesity and its related conditions such as MetS (Tou et al., 2011). Therefore, LNCS are promoted as a replacement for NS albeit its usefulness is reducing obesity and its health-related effects has not been fully established (Sylvetsky & Rother, 2016).

Metabolic syndrome is characterized by the presence of at least three metabolic dysfunctions; insulin resistance, impaired glucose tolerance, dyslipidaemia, hypertension, and central adiposity (Saljoughian, 2017; Kelli & Kassas, 2015). The metabolic effect of insulin resistance includes impaired glucose tolerance and ultimately T2D, hypertension, dyslipidaemia, visceral adiposity and elevated levels of inflammatory markers (Freeman & Pennings, 2021a). Dyslipidaemia is an imbalance in the body lipid levels, resulting in an elevated amount of total cholesterol (TC), low-density lipoprotein (LDL-C), triglycerides (TG) and reduction in high-density lipoprotein (HDL-C) (Nikkos & Rehman, 2021). Most people with dyslipidaemia also have concurrent hypertension due to their synergistic effect on each other (McEvoy et al., 2018).

Caloric sweeteners can induce lipogenesis in the liver which can result in insulin resistance and the build-up of fat in the abdominal region (central obesity) resulting in increased waist circumference. A clear continuous association has been shown between central obesity and clinical outcomes such as hypertension, dyslipidaemia and diabetes (Després et al., 2008). These risk factors interact with each other in the pathogenesis of the cardiometabolic syndrome.

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Research has shown that LNCS have an indirect effect on insulin production and glucose homeostasis through its effect on modulating the gut microbiota (Bueno-Hernández et al., 2020). With conflicting data between MetS and LNCS intake from observational research, the use of nutritive sweeteners in weight management is not supported by the evidence from randomised control trials. Whereas controlled trials and population-based research have linked free sugar consumption to the development of the MetS (Stanhope, 2016b) (Azad et al., 2017).

An experimental animal model involving rats (Sprague Dawley rats) was used to study the effect of commonly consumed natural sweeteners on insulin signalling pathway and the MetS. Specific focus was on commonly consumed sweeteners on the Ghanaian market, namely granulated white sugar, granulated brown sugar, honey and stevia. Rats are good models for studying the effect of sweeteners on glucose metabolism and the molecular mechanism underlying metabolic conditions that mimic what is seen in humans (Baena et al., 2016). To the best of my knowledge, this is the first study to look at the effect of various types and doses of sweeteners on metabolic health and insulin signalling via the GLUT4 pathway in one study.

1.2 PROBLEM STATEMENT

Metabolic syndrome is a disease of public and global health concern due to its associated morbidity, mortality and increased health care cost associated with its management and global economic impact. The incidence of MetS parallels an increase in the incidence of T2D and obesity (Saklayen, 2018a). In the United States (US), the weighted prevalence of MetS was 34.7%. Over a five-year period (2011-2016), the incidence among young persons (20-39 years) increased significantly, while the prevalence among those 60 and older remained higher (48.6%) (Hirode & Wong, 2020). In sub-Saharan Africa, the reported prevalence in Nigeria was 18% in the quasi-urban population and 10% in the rural community, nonetheless a cross-sectional analysis of a sample of the population in Ghana indicated a prevalence of 69% based

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on IDF criteria (Osei-Yeboah et al., 2017). However, Ofori-Asenso, Agyeman, & Laar, (2017) reported a prevalence of 21% according to the IDF criteria, contrary to the increased prevalence of 58% according to the IDF criteria in a resource poor community in urban Ghana (Christian et al., 2021)

Unhealthy dietary intake is a risk factor for cardiometabolic syndrome (CMS). Most notable among them is the excessive consumption of added sugars with the prevalence of CMS increasing chronologically and statistically with an increase in the consumption of sweeteners (Sun & Empie, 2012). Obesity, insulin resistance, poor glucose tolerance, dyslipidaemia, and hypertension have all been linked to an increase in the use of nutritive sweeteners (Hieronimus et al., 2020; Swithers, 2016; Tou et al., 2011). Dietary sugars have also been associated with the pathogenesis of chronic low-grade inflammation which is contributing factor in cardiovascular diseases and T2D development (Della Corte et al., 2018; Rosales-gómez et al., 2018). It has been reported that a diet pattern consisting of sweets or sweeteners helps with *de novo* synthesis of free fatty acids and its metabolites in the liver that can trigger an inflammatory response and the production of reactive oxygen species (Della Corte et al., 2018).

Based on the link between free sugar intake, dietary quality, and obesity, WHO has recommended a daily intake of free sugars of less than 10% of total energy intake (equivalent to 50 g of sugar or 12 teaspoons of sugar, translating to 200 kcal based on a 2000 kcal diet) and a further reduction to less than 5% (equivalent to 25 g of sugar or 6 teaspoons of sugar, translating to 100 kcal (World Health Organisation, 2018). Similarly, the 2020 – 2025 American Dietary Guidelines proposed that all Americans aged two and above restrict their added sugars to no more than ten per cent of total calories (U.S. Department of Agriculture and U.S. Department of Health and Human Services, 2020). The American Heart Association (AHA) also recommended that American men and women should not consume more than 150

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and 100 calories per day respectively from sugar-added foods or free sugars, to ensure good dietary habits in the prevention and treatment of lifestyle related conditions (Zhang et al., 2018). The average child, teenager or adult in the US consumes about 17 teaspoons of sugar which is equivalent to 14% of the total energy per day according to a 2000 kilo calorie diet (Zhang et al., 2018). Added sugar intakes are estimated to represent 15% to 21% of daily energy intake in adults and 16% to 26% in children in Europe, but added sugar accounts for 7% to 11% of energy intake in adults and 11% to 17% in children in the United Kingdom (Azaïs-Braesco et al., 2017). In tracking the dietary transition in Ghana, sugar and sweeteners have been recorded to have the greatest increase (1075 kcal) in consumption between 1983 and 2013 which parallels a corresponding increase in energy intake, over weight and obesity (Kushitor, 2021). Ghana's First National Food-Based Dietary Guidelines recommends reducing intake of added sugars (Ministry of Food and Agriculture & University of Ghana School of Public Health, 2023).

The evidence against the intake of caloric sweeteners has fuelled the production, promotion, and usage of LNCS. Unfortunately, several studies have associated cardiometabolic risk factors with increased usage of LNCS although the evidence remains inconclusive (Swithers, 2016) (Romo-Romo et al., 2016)). The absorption of LNCS do not occur in the small intestines and instead, pass into the large intestines where they are eliminated serving as a substrate for microbiota located in the large intestines inducing dysbiosis of the gut microbiome inducing the production of pro-inflammatory cytokines (Mattes et al., 2021; Satokari, 2020). Low or No Calorie Sweeteners have the ability to interact with the taste receptor family causing a release of neurotransmitters and hormones which have negative metabolic effects (Liauchonak et al., 2019).

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The production of granulated white sugar, sugar cane and or sugar beets undergo various purification processes resulting in the production of by products including non-centrifugal cane sugars, brown sugar, and molasses. These breakdown products in sugar refining have been shown to contain bioactive substances such as flavonoid glycosides and phenolic acids with favourable health effect as compared with normal white sugar (Castro-Muñoz et al., 2022). Honey is also postulated to be more nutritive than granulated white sugar due to its low glycaemic index and presence of carbohydrates, amino acids, enzymes and phytochemicals (Bobiş et al., 2018a).

Policy recommendations also group sweeteners together, suggesting that all sweeteners have a similar effect on the body. However, sweeteners have varying constituent sugars and unique chemical structures hence may exert a distinct effect on insulin signalling and metabolism, which will consequentially determine their suitability for normal and diabetic persons. Numerous epidemiological researches have indicated the intake of free sugars either as part of packaged foods or to cooked foods and sugar sweetened beverages skyrocketed over the years. Despite national policies and public health interventions to reduce consumption of sugar sweetened beverages (SSB) their intake still remains higher than the recommendation for the intake of added sugars (Malik & Hu, 2022a). This increase in the consumption despite efforts such as SSB taxes is observed largely in low- and middle-income countries as a result of the nutrition transition(Malik & Hu, 2022a). In Africa very few countries (Morocco, Mauritius, South Africa, Seychelles) have introduced taxation on SSB (Popkin & Ng, 2021). Based on the NHANES data SSB remain the major source of added sugars followed by sweetened baked products, refined sugars (white and brown sugar), syrups and toppings (DiFrancesco et al., 2022).

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Experimental studies investigating the individual markers of MetS have focused more on fructose corn syrup or individual sugars. Currently no research has compared in one study the effect of different types of nutritive and non-nutritive sweeteners on the different components for MetS. Also, the molecular processes mediating the adverse consequences of the individual sweeteners via the GLUT4 pathway and hexokinase II expression have not been explored.

1.3 SIGNIFICANCE OF STUDY

The study will give information on how different doses and types of natural sweeteners (NS and LNCS) affect insulin signalling via the GLUT4 signalling pathway, determining which sweetener is better for cardiometabolic health. This study will build on efforts by offering strategies, such as lifestyle changes in recommending the type and quantity of sweetener to consume based on an individual's metabolic health. It will also contribute to addressing anecdotal evidence or testimonials from the public regarding which sweetener is healthier. Changes in lifestyle modification will cause a decrease in the prevalence of cardiometabolic conditions related to the high intake of sweeteners and their products and a reduction in health care costs and the economic burden associated with it. Thus, the conjecture on caloric versus non or low-caloric sweeteners and the health benefits will be put to a test which will consequentially determine their suitability for health.

1.4 HYPOTHESES

H_0 – There is no significant difference in the glucose signalling pathway among the different sweeteners.

H_0 – There is no significant difference in the effect of the various sweeteners on cardiometabolic risk factors.

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1.5 AIM AND SPECIFIC OBJECTIVES

1.5.1 Aim

This research aimed at determining the effect of the intake of commonly consumed natural sweeteners on nutrition, glucose signalling pathway, and metabolic risk factors using Sprague Dawley rats.

1.5.2 Specific Objectives

The specific objectives of the study were to:

1. determine the effect of sweeteners on energy intake, food efficiency, weight and relative organ weight.
2. assess the effect of sweeteners on cardiometabolic risk factors (lipid profile and lipid ratios, fasting plasma glucose, serum insulin, insulin resistance).
3. examine the effect of sweeteners on GLUT4 protein concentration.
4. examine the effect of sweeteners on glucose metabolic pathway by hexokinase II.
5. examine the effect of sweeteners on the histology of the pancreas, skeletal muscle, and brain.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 SWEETENERS

2.1.1 Nutritive and Non-nutritive

Sweeteners remain useful in the food and pharmaceutical sectors. Aside from enabling good taste in cooked and manufactured foods, they confer other useful properties such as inhibiting microbial growth in jams and jellies. Furthermore, they add texture, flavour and colour to confectionaries and baked foods, supporting the growth of yeasts in fermentation or leavening, contributing to the volume of foods such as ice cream, balancing the acidity in salad dressings, condiments and sauces among others (Fitch & Keim, 2012). In the pharmaceutical industry, sweeteners have remained useful by adding bulk and consistency to medications in addition to masking their unpleasant tastes to make them palatable for consumers ((Shah, Jain, Laghate, & Prabhudesai, 2020). Despite the multifunctional nature of sweeteners, their health implications cannot be underestimated.

Sweeteners can be categorized in different ways. However, for the purpose of this study sweeteners will be categorized into two groups:

a. Nutritive sweeteners (NS) or caloric sweeteners (CS)
b. Non-nutritive sweeteners (NNS) or low/no calorie sweeteners (LNCS).

In this review the synonyms within each category of sweetener will be used interchangeably. Nutritive sweeteners are defined as sweeteners that contain carbohydrates and provide energy (~4 kcal/g) after consumption. Non-nutritive sweeteners, contrarily, add very few calories or none at all when consumed (Fitch & Keim, 2012; Tou et al., 2011).

Nutritive sweeteners are found naturally in foods or are incorporated during the production process. Nutritive sweeteners can be naturally occurring (for example, lactose in milk and other

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dairy goods and fructose in fruits) or added sugars that are not naturally present in foods but are added during preparation, preservation, or production. White granulated sugar, brown sugar, honey, fructose corn syrup, maple syrup and agave nectar are some examples of added sugars (Fitch & Keim, 2012). They consist of simple sugars, monosaccharides, and disaccharides. Monosaccharides are the simplest forms of carbohydrates which require no further digestion by the intestinal brush border enzymes and are easily absorbed. Nutritive sweeteners contain nutritionally important monosaccharides like glucose, fructose, and galactose. Disaccharides are made up of two monosaccharide molecules that are covalently bound together. Lactose (glucose and galactose), sucrose (glucose and fructose), and maltose (glucose and glucose) are common disaccharides. However, unlike sucrose and maltose which can be found in added sugars, lactose is found in milk and milk products (Binder & Reuben, 2017; Tou et al., 2011). The different NS may have either one type of sugar or a combination of sugars in different proportions. Non-nutritive sweeteners on the other hand offer little or no energy when consumed (Plaza-Diaz & Gil, 2015). Examples of some approved NNS's include aspartame, acesulfame-potassium, neotame, saccharin, sucralose, advantame, stevia and monk fruit (Shum & Georgia, 2021a).

2.1.1.1 Granulated white sugar

Technological advancement in the 18th century resulted in the production of granulated white sugar (Edwards, Rossi, Corpe, Butterworth, & Ellis, 2016) commonly referred to as "table sugar" or "white sugar". White sugar is an extract of sugar cane *(Saccharum officinarum L.*) and sugar beets (*Beta vulgaris L.*) produced by two separate processes. Sugar cane and sugar beets are processed into raw sugar, which is then refined into granulated sugar. This is done by the extraction and purification of the pure sugar juice followed by concentrating and crystallizing the soluble sucrose (Wojtczak et al., 2012). White sugar is readily available with a relatively low cost in production (Edwards et al., 2016; Tou et al., 2011). Sucrose is the main

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carbohydrate found in table sugar. Although sucrose is the body's major source of energy, it can have a negative impact through the development of metabolic disorders such as impaired glucose and lipid metabolism (Castro-Muñoz et al., 2022).

2.1.1.2 Granulated brown sugar

The processing of raw sugar to refined sugar reduces its nutritional value by eliminating bioactive molecules such as flavonoid glycosides and phenolic acids which led to the recommendation for the production of less refined derivatives such as brown sugar (Castro-Muñoz et al., 2022). Granulated brown sugar has crystals that are comparable to that of white granulated sugar but have a pale-yellow tint due to small amount of molasses present (Chen et al., 2021). The presence of molasses gives it a distinct flavour.

Granulated brown sugar is known by various names, including 'Jaggery' in India, 'Panela' in Latin America, and 'Kokuto' in some Asian nations such as Japan. Minimally processed sugar like brown sugar is also termed non-centrifugal sugar by the Food and Agricultural Organization (Zidan & Azlan, 2022). Granulated brown sugar is produced by first extracting the sugarcane juice, followed by evaporation of the juice to form a syrup. The syrup undergoes vigorous stirring and moulding to form crystals from which brown sugar is obtained (Chen et al., 2021). Granulated brown sugar is composed of monosaccharides (glucose and fructose), a disaccharide (sucrose), polyphenols and flavonoids. Additionally, it is refined to a lesser extent compared to granulated white sugar (Lee et al., 2018).

2.1.1.3 Honey

An inclination towards healthy eating has resulted in a shift from the intake of refined sugars to more natural, healthier and medicinal alternatives such as honey, dates and jaggery (Arshad et al., 2022). Honey is an ancient natural sweetener produced by bees from the nectar or secretions of plants. It has remained effective in both traditional healing and modern medical

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practices (González-Montemayor et al., 2019). Honey is one of the most sought-after nutritive sweeteners as a result of its biologically active substances which are the phenolic compounds, flavonoids, carotenoids, minerals, vitamins, enzymes (glucose oxidase, diastase, and invertase), and aromatic substances (González-Montemayor et al., 2019). It is known to possess anti-inflammatory, antibacterial, immunostimulatory, antiviral, wound repair, anti-diabetic, and cholesterol-lowering effects (Bobiş et al., 2018b; Hossain et al., 2020).

Honey's chemical composition is determined by factors such as the place of origin of the plant from which the nectar was derived, environment and climatic conditions and the processing procedures (Erejuwa, Sulaiman, & Ab Wahab, 2012; González-Montemayor et al., 2019). Honey contains 75% monosaccharides (glucose and fructose), 15% disaccharides (sucrose), small quantities of oligosaccharides and bioactive compounds, and 17-20% water (P. M. Da Silva et al., 2016; González-Montemayor et al., 2019). Despite its therapeutic effect, its caloric addition to foods cannot be overlooked.

2.1.1.4 Stevia

The development of non-caloric high-intensity sweetening agents as alternatives to caloric sweeteners has led to an increase in their production and variety. Stevia is a non-caloric sweetener extracted from the leaves of *Stevia rebaudiana* (Bertoni), which has over 200 species worldwide. It has been used as a sweetening and flavouring agent for over 100 years. It was originally consumed by the natives of South America by drying its leaves and used to sweeten beverages and medications or chewed as a treat (Ashwell, 2015; Castro-Muñoz et al., 2022). It has been nicknamed; honey leaf, candy leaf or sweet leaf due to its sweetness (~100 – 400 times higher than sucrose) (Mlambo et al., 2022).

Stevia has its sweet taste originating from its sweet tasting component, steviol glycosides. About 11 steviol glycosides have been identified. These are stevioside, Rebaudioside A,

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Rebaudioside B, Rebaudioside C, Rebaudioside D, Rebaudioside E, Rebaudioside F, rebusoside, steviolmonoside, steviolbiocide, and Dulcoside A. The glycosides consist of a steviol back bone attached to glycosidic units.

In addition to glycosides, stevia contains vitamins, minerals, essential amino acids, fatty acids, and bioactive substances (flavonoids, phenolic compounds, phytosterols, chorogenic acids, triterpenes, hydrocarbons, and crude fibre) (Ahmad, Khan, Blundell, Azzopardi, & Mahomoodally, 2020; Schiatti-Sisó, Quintana, & García-Zapateiro, 2022). Stevia is a generic name used to refer to different forms of the stevia sweetener. Commercially it is available in four different forms; (a) as pure stevia extract, (b) as a sugar-transferred stevia extract, (c) as Reb A-enriched extract which have been used in a number of foods and beverages and (d) combined preparation of stevia sweeteners.

In order to reduce its unpleasant aftertaste, the pure stevia extract is enzymatically modified to sugar-transferred stevia extract and Rebaudioside-A enriched stevia extract which tastes like sucrose. These improved forms of the stevia extract are used in the beverage industry, in confectionery, ice-cream, dairy products and as table top sweeteners (Mizutani & Tanaka, 2001). Commercially available pure stevia extracts contain about 90% of steviol glycosides, whereas the sugar transferred, and rebausdioside-A enriched stevia have lesser steviol glycoside content than the pure extracts. Combined preparations of stevia are produced by mixing one of the basic forms of stevia with dextrin or lactose to be used as a bulking agent in food manufacturing. It is also combined with sugar alcohols such as erythritol and maltitol used in sugarless gum, candies and chewable containing Vitamin C (Mizutani & Tanaka, 2001). Studies have associated stevia intake with numerous health benefits. However, these studies focused on stevia extracts with high stevia glycoside concentration. There is a paucity of data

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on commercially modified stevia extracts being used in the food industry and as a table top sweetener.

2.1.2 Trends in Production and Consumption

Sugar has become an essential commodity worldwide and is consumed daily due to its usage in the food and beverage industry (Abdullah et al., 2015). Production of white sugar from sugar cane accounts for 80% of the world's sugar production with majority of the output from Mexico, India, Thailand, Brazil, China, US, Russia, Pakistan, France and Australia (International Sugar Organization, 2019). Between 2001 and 2018, global sugar output scaled from 123.454 million tonnes to 172.441 million tonnes, which represented a 2% yearly increase. This growing demand for sugar is typically driven by a growth in the world's population, per capita income and growing health concerns among others (International Sugar Organization, 2019).

2.1.2.1 Trends in the production of white and brown sugars

According to the United States Department of Agriculture, sugar remained one of Ghana's top ten imported foods in 2022 (USDA, 2022). Global production of brown sugar parallels the growth in the food and beverage and bakery industry, and it has been forecasted to grow rapidly. Globally, Brazil is the main producer of sugar (OECD/FAO, 2021). Over the last decade, 22% of the global sugar production was from developed countries (OECD/FAO, 2021). Between the period of 2017 and 2018, white sugar exports constituted 34% of global trade (OECD/FAO, 2018).

2.1.2.2 Trends in the production of honey

Globally, the demand for honey has surged over the last 20 years as the world population continues to grow. Current dietary guidelines have recommended limiting the intake of added sugar for better health. COVID-19 has also led to a demand for immunity-enhancing foods and

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supplements in comparing pre-pandemic demand to post-pandemic demand (García, 2018; Ribeiro et al., 2019). The increased global demand has translated to an increase in global exports of honey. From 2010 to 2020, the production of beehives worldwide increased from 80 million beehives to 94 million beehives with the global honey production reaching a peak volume of 1.88 million metric tons (Shahbandeh, 2021). Between 2019 and 2020, the demand for honey increased by 9 %. In 2021 the global market size was USD 8.15 million which increased to USD 8.53 million in 2022 and it is expected to reach USD 12.69 million in 2029 (Shahbandeh, 2021).

Honey is popular in the ethnic cuisine in Japan and South Korea. These two countries depend on the importation of Western honeybees because they produce more honey than Japanese honeybees, even though Japanese honeybees are more disease resistant. Between 1994 and 2005 honey importation in South Korea had increased from under 50,000 kg to 1.2 million kg due to failure of the local honey production to thrive (Kohsaka et al., 2017). In assessing the consumption habits and determining factors for honey consumption in Portugal, it was reported that honey was being consumed once a week or once a month usually during winter and autumn for its medicinal purposes in treating cough and other respiratory illnesses during those seasons (Ribeiro et al., 2019).

2.1.2.3 Trends in consumption

White granulated sugar, brown sugar, honey, and stevia are consumed as table top sweeteners or as added sugars or in food manufacturing and processing. Many populations are consuming added sugar beyond the recommendation by WHO with a growth observed in low- and middle-income countries (Gillespie et al., 2023a; World Cancer Research Fund International, 2015). The lowest sugar consumption is in Sub-Saharan Africa whereas the regions of the Middle

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East, Northern Africa, and Western European countries have the highest (World Cancer Research Fund International, 2015).

The relatively low cost in producing them makes them cheap and available (World Cancer Research Fund International, 2015). Examining the emerging patterns of added sugars and their sources among children, adolescents, and teens in US, (Ricciuto, Fulgoni, Gaine, Scott, & Difrancesco, 2022) reported a decline in the consumption of added sugars from sugar sweetened beverages between 2001 – 2018. However, the consumption of added sugars from coffee and tea increased.

In spite of the observed decline, the quantity consumed still remains above the recommendation by the Dietary Guidelines for Americans (10% of energy per day). A related study was carried out in the United States among young (19-50 years) and older (50+ years) persons using NHANES data from 2001 to 2018. A decrease in added sugar intake was observed in younger adults (19-50 years) but not in older adults (50+ years), which was attributed to the fact that older adults tend to generally take less added sugars, whereas younger adults tend to consume more and thus have an increased likelihood of reducing intake (DiFrancesco et al., 2022).

Between 2017 and 2018, the average daily intake of sugar from added sources among US children and young adults was 17 teaspoons, with older persons aged 20 years and above having a comparable intake (Centers for Disease Control and Prevention, 2021). The observed decline in added sugars was due to a significant reduction in the intake of sugar sweetened beverages as a main contributor to the intake of added sugar although there was an increase in the intake of added sugar from tea and coffee. This observed decline has been credited to policies targeting sugar sweetened beverage consumption by reformulating products to use LNCS, taxing sugar sweetened beverages, public health education encouraging the intake of water as a healthier alternative (Popkin & Ng, 2021; Vargas-Garcia et al., 2017). A key health

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indicator for the "Healthy People 2030" "is to reduce consumption of added sugars for individuals aged 2 years and above" (Centers for Disease Control and Prevention, 2021).

In Europe, added sugars contributed to 11 – 17% of the total energy intake in children and 7 – 11% in adults with and the majority of the population, particularly children exceeding the recommended intake by WHO (Azaïs-Braesco et al., 2017). In Eastern Europe, a decline was observed in the intake of sugar sweetened beverages among adolescents between 2002 and 2018 with the highest decline observed among the highly affluent (Chatelan et al., 2021).

There is limited data on the trend and consumption pattern of added sugars and sugar sweetened beverages in Africa. In South Africa, the consumption pattern has been estimated between 60 - 120 grams of sugar daily with 20 - 80 grams obtained from sugar sweetened beverages (McCreedy et al., 2022).

2.1.3 Digestion and Metabolism

Sweeteners are digested and metabolized after consumption just like any other edible product. Upon ingestion of white granulated sugar its main constituent, sucrose, is hydrolysed into its glucose and fructose by the enzyme sucrase an intestinal brush boarder enzyme. About quarter (25%) of the fructose is further converted to glucose (Anderson, 2003). Absorption of the monosaccharides involves movement from inside the small intestines, across the intestinal cells and into the blood. The sodium coupled glucose transporter SGLT-1 (Fig. 2.1), located at the microvilli of the small intestine, transports glucose from the intestinal lumen into the epithelium. The transmembrane sodium gradient maintained by the sodium/potassium pump facilitates the movement of glucose into the epithelium. Fructose transport from the lumen into the epithelium on the other hand is facilitated by the glucose transporter GLUT 5 (Fig. 2.1) (Anderson, 2003; Kohlmeier, 2013; Wright et al., 2018a). In the

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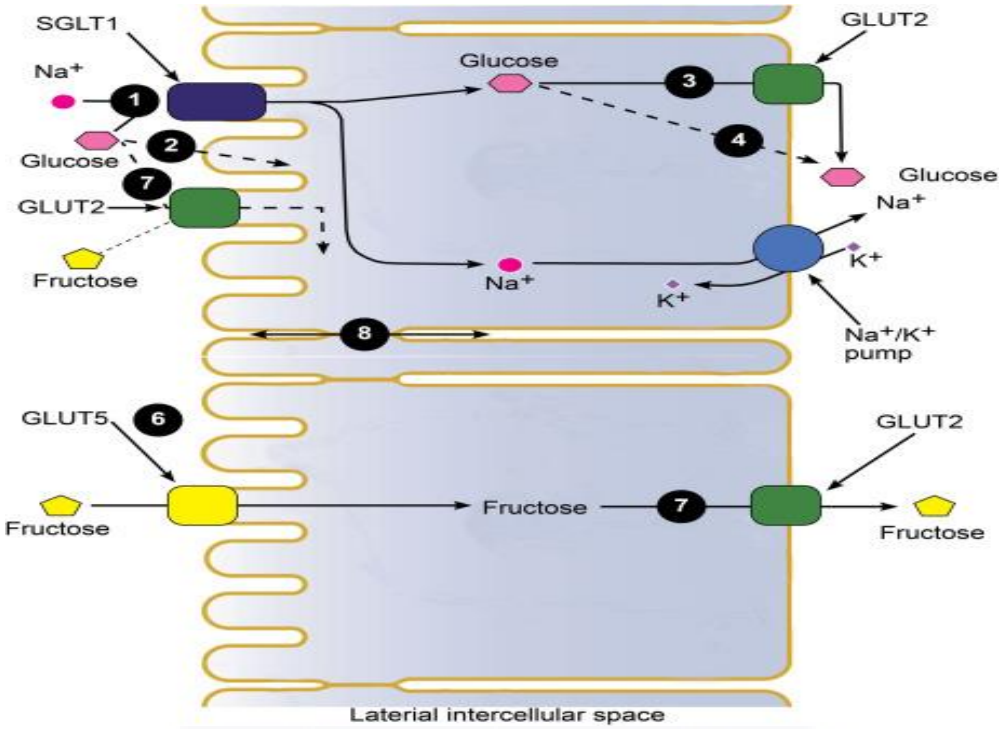

Figure 2.1: Glucose and fructose absorption across the intestinal epithelium (Wright et al., 2018b). Glucose moves from the intestinal lumen into the epithelium by SGLT1. Fructose moves into the epithelium by GLUT5. GLUT2 transports both the glucose and fructose to the basolateral membrane where it is transported by the portal vein.

intestinal epithelium both fructose and glucose are absorbed into the blood via a facilitated glucose transport protein GLUT2 (Wright et al., 2018a).

The portal vein transports glucose and fructose into the cells of the liver for further metabolism. In the hepatocytes, fructose is phosphorylated to fructose-1-phosphate and triose-phosphates by the enzymes fructokinase. Phosphorylated fructose is further oxidized by the hepatocytes to produce lactate and glucose which either moves back into the blood or contributes to the glycolytic pathway.

A significant amount of the phosphorylated fructose also contributes to the *de novo* lipogenesis producing triacylglycerol which has been associated with the adverse effect of fructose intake (Gibson et al., 2013; Tappy et al., 2010). On reaching the liver, fructose is either transported back into the circulatory system facilitated by GLUT2 or stored in the hepatocytes as glycogen

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(Wright et al., 2018b). Sucrose present in brown sugar undergoes the same digestion and absorption process observed with white sugar intake. Both glucose and fructose are transported at the apical membrane via SGLUT-1 and GLUT-5 and at the basolateral membrane via GLUT-2 (Wright et al., 2018a).

Aside the monosaccharides in honey, other di-, tri- and tetra- saccharides have been identified with the disaccharides (maltose and sucrose) constituting about 10-15% (Lazarević et al., 2017). The brush border enzymes maltase breaks down maltose to glucose units and sucrase digests sucrose to glucose and fructose (Xu et al., 2012). The monosaccharide units (glucose and fructose) are absorbed into the enterocytes by SGLUT-1 and GLUT 5 for glucose and fructose, respectively (Anderson, 2003). The high fructose content of honey (21 – 43%) has been linked to its low glycaemic index and hypoglycaemic effect. Although the exact mechanism remains unclear, it has been proposed that the high fructose reduces the rate at which the stomach empties its content into the small intestines, slows down intestinal uptake, suppresses appetite and activates the action of glucokinase in the hepatocytes which plays a key role in the uptake and storage of glucose as glycogen. (Bobiş et al., 2018b). Several *in vitro* studies have assessed the bio-accessibility, bioavailability and consequently on the bioactivity of the phenolic compounds in honey (Alevia et al., 2021) with less focus on the sugars.

Stevia's active compound is the steviol glycoside. Following the intake of stevia, steviol glycoside passes through the upper gastrointestinal tract without undergoing digestion. However, in the colon the steviol glycosides are hydrolysed by colonic microbiota, cleaving the glycosidic bonds thereby removing the sugar or the glycoside unit from the steviol backbone. The glycoside is transported to the liver for glucuronidation to steviol glucuronide and excreted via urine in humans and faeces in rats (Ajami, Seyfi, Abdollah, et al., 2020; Ashwell, 2015; Samuel et al., 2018). As a result, pure stevia extract does not contribute to the

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rise in blood glucose level when added to a meal. However, the highly processed stevia products which are compounded together with other bulking or sweetening agents may have a different digestive and metabolic pathway from the pure stevia extract which needs to be explored.

2.2 METABOLISM OF GLUCOSE AND FRUCTOSE

Glucose and fructose are the main end products absorbed into the general circulation by the portal vein after digestion of granulated white sugar, granulated brown sugar and honey. Carbohydrates obtained from dietary sources affect metabolism in various ways such as; the type of the monosaccharide, the quantity absorbed, the rate of its absorption and the breakdown by colonic bacteria (Wolever, 2003).

2.2.1 Metabolism of Glucose

Glucose is an indispensable source of energy in humans, with every cell in the human body utilizing it for energy. It is referred to as the "energy transport system" in humans, with the body organs and systems like the central nervous system depending on it for energy (Navale, 2019; Von Rymon Lipinski, 2006; Yang, 2014). The rate of glucose entry balanced by the rate of glucose clearance from the bloodstream determines the blood glucose level. Plasma glucose concentration is chiefly maintained through intake of carbohydrate foods or sources (Aronoff et al., 2004; Decubellis & Mayer, 2013). Glucose obtained from the intake of sweeteners is readily absorbed by the enterocytes after which it enters the general circulation via the hepatic portal vein thus raising the blood glucose concentration with excess glucose stored in the liver as glycogen.

Maintaining an adequate amount of glucose level in the blood is required not only for survival, but also for maintaining good health. High concentration of glucose in the blood stream is an indicator of poor metabolic health and a key symptom of diabetes mellitus (Banday et al.,

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2020). Dysregulated hyperglycaemia is a precursor for organ and tissue damage due to glucotoxicity. In contrast uncontrolled hypoglycaemia can culminate in death and coma (Kawahito et al., 2009). The balance between glucose entry and clearance from the blood is chiefly maintained by the action of insulin and glucagon (Yang, 2014).

High postprandial glucose level is achieved through the production of insulin by the pancreas, stimulating an increase in the absorption of glucose by other tissues and organs such as the skeletal muscle, adipose tissue, and the brain (Aronoff et al., 2004). In addition, insulin stimulates hepatic uptake of glucose, promotes glycogen synthesis (glycogenesis) and prevents the production of glucose from glycogen and non-carbohydrate stores by inhibiting the release of the hormone glucagon (Aronoff et al., 2004; Jiang et al., 2020). Glucagon on the hand has an antagonistic effect on insulin action. It maintains glucose homeostasis especially during fasting states by stimulating glycogenesis and gluconeogenesis (Aronoff et al., 2004). However, in individuals with type II diabetes, insulin is unable to inhibit glucagon secretion. As such, there is continuous hepatic glucose production resulting in imbalance between glucose appearance and glucose disappearance (Aronoff et al., 2004).

When blood glucose rises, insulin produced from the beta cells stimulates the production of glucokinase in the liver. Glucokinase catalyses the phosphorylation of glucose to glucose-6-phosphate (G6P) (Fig. 2.2) (Adeva-Andany et al., 2016; Rui, 2014). In other body tissues such as the muscle, red blood cells and the brain, hexokinase instead of glucokinase is responsible for the phosphorylation of glucose to G6P (Adeva-Andany et al., 2016). Glucose-6-phosphate is transformed into either glucose-1-phosphate, fructose-6-phosphate or oxidised into gluconolactone. Glucose-1-phosphate is the precursor for the formation of glycogen, while fructose-6-phosphate is the precursor glycolysis and production of fatty acids (Fig. 2.2) (Adeva-Andany et al., 2016).

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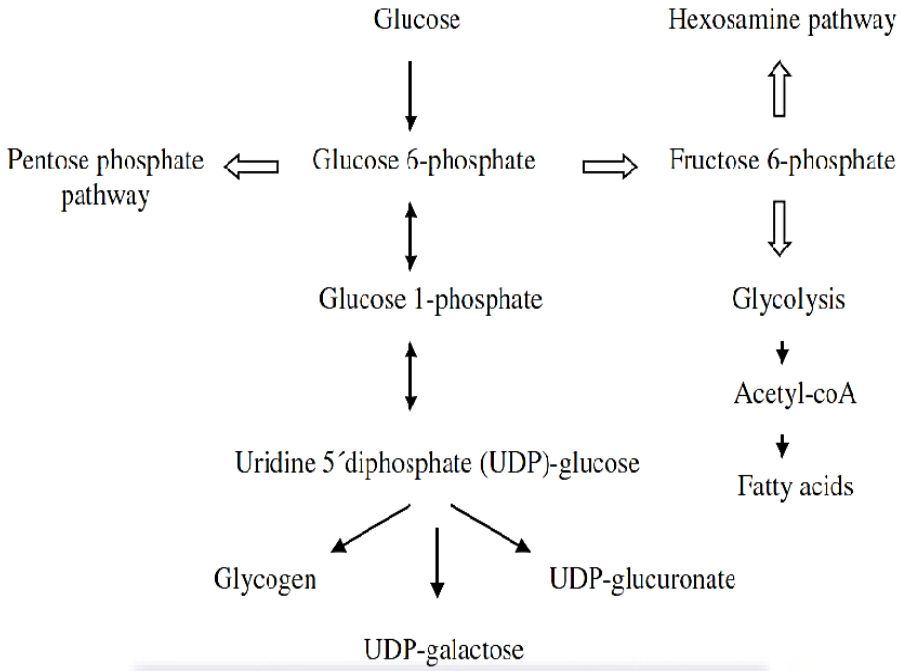

Figure 2.2: Glucose metabolism in the liver (Adeva-Andany et al., 2016) This diagram provides the various metabolic pathway of glucose in the liver. Glucose is concerted to Glucose-6-phosphate which either enters the pentose pathway, glycogenesis pathway or the hexosamine pathway for fatty acid synthesis.

Glucose-1-phosphate is converted to UDP-glucose, which is needed for the synthesis of glycogen, UDP galactose, and UDP-glucuronate. Fructose 6 phosphate may either enter the hexosamine pathway or in glycolysis to produce pyruvate and acetyl-coA (Fig. 2.2) (Adeva-Andany et al., 2016).

The glucose transporter GLUT4 facilitates glucose absorption in skeletal muscle and adipose tissue. Insulin attaches to an insulin receptor (IR) in the skeletal muscles, causing tyrosine kinase to be phosphorylated. Tyrosine kinase recruits different substrates such as the insulin receptor substrate (IRS). Phosphorylated IRS engages the p85 subunit of PI3K and the p110 catalytic subunit, resulting in a rise in phosphatidylinositol -3,4,5 triphosphate. As a result, the protein kinase Akt is activated, and Akt substrate 160 (AS 160) is phosphorylated, allowing GLUT4 to move to the cell membrane of the muscle cells for glucose to enter the cells (Yang, 2014). The skeletal muscle is vital in maintaining glucose homeostasis through its insulin-

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mediated glucose uptake through the glucose transporter GLUT4. Following the influx of glucose into the cells by GLUT4, hexokinases catalyse the preliminary step of glucose metabolism by phosphorylating glucose in the intracellular compartment to produce glucose-6-phosphate, which serves as a precursor for glycolysis, pentose phosphate pathway, glycogenesis, and the hexosamine biosynthetic pathway (Roberts & Miyamoto, 2015a).

2.2.2 Metabolism of Fructose

Fructose is one of the three naturally occurring monosaccharides found in humans. It is commonly found in honey, fruits (apple, grapes, banana, orange, plum, prune, pear) and vegetables (tomato, onion, white cabbage, lettuce, leek, asparagus) either together with glucose to form sucrose as present in cane sugar or it can exist alone in its free form. Fructose can also be found in other non-natural sources such as fructose corn syrup often used in the food and beverage industry (Annandale et al., 2021; Rumessen, 1992). It has also been found to be the sweetest among sugars, hence its use in the commercial production of sweeteners (Rumessen, 1992). The body has very little biological need of fructose as compared to glucose because majority of the cells are not capable of metabolizing fructose directly (Bray, 2007; Dholariya & Orrick, 2022). However, fructose promotes the uptake of glucose by the hepatocytes. It also helps to speed up the burning of carbohydrate stores and sustains hepatic gluconeogenesis by providing energy for use by the skeletal muscle in times of strenuous physical activity (Dholariya & Orrick, 2022).

Fructose is passively absorbed in the small intestine's apical membrane by GLUT-5, which has a high affinity for fructose (K_m = 6 mM). The intestine of a healthy adult is capable of absorbing fructose within a range of 5 g to 50 g. Unabsorbed fructose in the distal part of the small intestines and the colon is acted on by intestinal bacteria resulting in fermentation which produces gas and other metabolites responsible for symptoms such as bloating and abdominal

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pain and discomfort (Hannou et al., 2018). Once fructose enters into the portal circulation, almost all of it is rapidly absorbed by the liver to maintain a peripheral plasma fructose concentration of 0.01 mM to 0.04 mM (Bray, 2007; Hannou et al., 2018). The liver is essential for metabolizing fructose in the human body.

Three key enzymes are involved in the fructose metabolism in the liver; fructokinase, aldolase B or fructose bisphosphate aldolase B and triokinase also known as ATP dependent dihydroxyacetone kinase (Campbell et al., 2014). Fructokinase catalyses the conversion of fructose to fructose-1-phosphate. Aldolase B catalyses the conversion of Fructose-1-phosphate to dihydroxyacetone phosphate (DHAP) and glyceraldehyde. Glyceraldehyde is phosphorylated to glyceraldehyde-3-phosphate (G3P) and acetyl coA. Both DHAP and G3P enter the glycolytic and gluconeogenic pathways (Fig. 2.3) (Dholariya & Orrick, 2022).

The liver converts DHAP to fructose 1,6 bisphosphate. Fructose 1,6 bisphosphate enters the glycogenic pathway to produce either glucose or glycogen. Due to the absence of a rate limiting step in fructose metabolism, high intakes of fructose will lead to an increased amount of the triose phosphates (G3P and DHAP) (Fig. 2.3) (Dholariya & Orrick, 2022). Increased amount of G3P and DHAP will stimulate the *de novo* lipogenesis and triacylglycerol synthesis (Dholariya & Orrick, 2022; Hannou et al., 2018; He et al., 2021).

Fructose metabolism in extrahepatic tissues is catalysed by the enzyme hexokinase which has a very low affinity for fructose as compared with its high affinity for glucose. Hexokinase converts fructose to fructose-6-phosphate which enters the glycolytic pathway (Dholariya & Orrick, 2022)(He et al., 2021). It has previously been proposed that metabolism of free form

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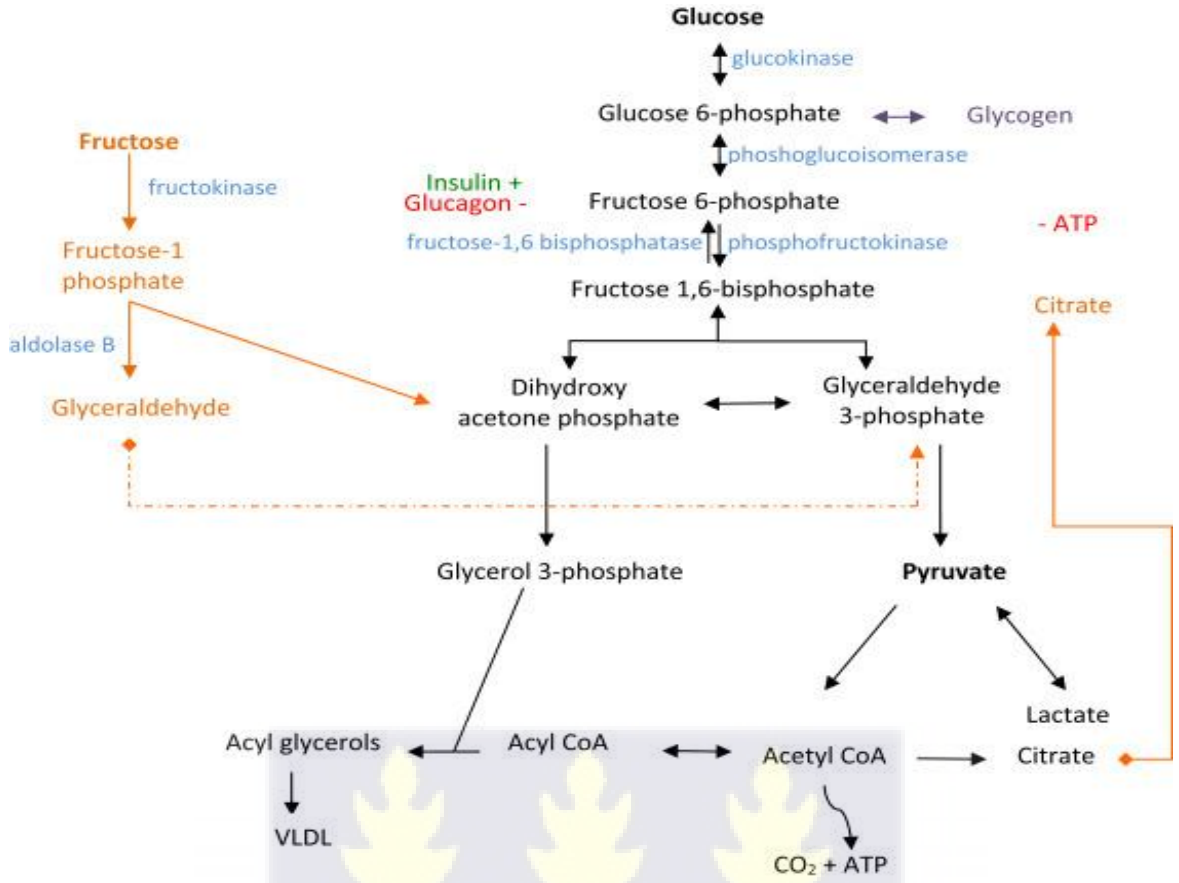

Figure 2.3: Hepatic fructose utilization (Stylianopoulou, 2022)**.** Fructose in the hepatocytes is phosphorylated to fructose-1-phosphate. Fructose-1-phosphate is converted to DHAP and G3P. They both enter the gluconeogenic and glycolytic pathway.

fructose such as is found in high fructose corn syrup is different from metabolism of bound fructose in sugars such as white granulated sugar, granulated sugar, and honey. However studies conducted in healthy and diabetic patients do not support this hypothesis (Tappy & Le, 2010).

There are important differences between the metabolism of glucose and fructose. Glucose metabolism has a rate limiting step controlled by fluctuation in plasma glucose concentration, cell energy level and insulin whereas fructose metabolism does not require or trigger an insulin response and lacks an inhibitory action (Campbell et al., 2014; Stylianopoulou, 2022).

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2.3 GLUCOSE AND FRUCTOSE TRANSPORT

The transport of carbohydrates in the body is regulated by two main groups of transporters which are the sodium linked glucose transporters (SGLUTs) and glucose transporter family (GLUTS) (Navale & Paranjape, 2016). The GLUT family consists of 14 members (GLUT1 - GLUT14), which have been grouped into three major classes (Class I, Class II, and Class III) based on similarities in their sequence and their choice of a substrate. There are six sodium-dependent glucose transporters (SGLT1 - SGLT6) (Sano, Shinozaki, & Ohta, 2020a). The main transporters involved in the transport of fructose and glucose as the main end products in the digestion of granulated white sugar, granulated brown sugar and honey are SGLUT1, GLUT2, GLUT5, GLUT4 (Navale & Paranjape, 2016).

Glucose is a commonly used substrate by the human body for energy production and molecular signalling. Given its polarised nature and large molecular weight, it is difficult to travel through lipid cell membranes via simple diffusion, thus it is assisted by transport proteins known as glucose transporters (Navale & Paranjape, 2016). Three (3) major transport proteins regulate glucose transfer throughout the body; SGLT-1, GLUT2 and GLUT4. Fructose transport in the body is mainly via the GLUT-5 (Navale & Paranjape, 2016).

2.3.1 Sodium Coupled Glucose Transporter 1 (SGLT1)

The sodium coupled glucose transporter-1 (SGLT1) belongs to a group of transporters referred to as the sodium linked glucose co-transporters (SGLTs) which are encoded by the gene SLC5A on chromosome 22q13.1 (Sano et al., 2020b). SGLT1 was the first among the SGLTs to be discovered and studied extensively. It has 14 transmembrane α-helical domains with -COOH and -NH_2 located in both the intracellular and extracellular spaces (Navale & Paranjape, 2016). SGLT1 has a high affinity for glucose and galactose. SGLTs transfer glucose and sodium in the same direction by transporting two sodium ions through SGLT1 for each glucose

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molecule by utilizing the sodium concentration gradient generated by the sodium-potassium pump (Navale & Paranjape, 2016; Sano et al., 2020b). SGLT1 is found at the microvilli of the small intestines and is responsible for glucose and galactose uptake from the lumen of the intestines through the enterocytes. SGLT1 is also present in other body tissues such as the kidney, brain, heart, trachea, testes and the prostate (Navale, 2019; Sano et al., 2020b).

A variation in the gene encoding for the SGLT protein results in an autosomal recessive order called glucose-galactose-disorder (Alruwaili & Alshdayed, 2023). Studies have also shown that, for diabetic individuals, brush border activity of SGLT1 increases by 3-fold which has also been shown to be correlated with high insulin levels. Increased SGLT1 activity indicates increased glucose absorption from the digestive system to the portal circulation (Navale & Paranjape, 2016).

2.3.2 Glucose Transporter 2 (GLUT2)

GLUT2 is a glucose transporter isoform belonging to a group of transporters referred to as the facilitative glucose transporters or GLUTs. The GLUTs unlike the SGLTs have 12 transmembrane helices with an amino acid group and carboxyl terminals located intracellularly. There are 14 identified GLUT isoforms encoded by the SLC2 gene (Navale, 2019). Their transport mechanism across cell membranes is by facilitated diffusion.

GLUT2 isoform is expressed in the pancreatic beta cells, hepatocytes, basolateral membrane of the small intestines, the kidney's epithelial cells and the central nervous system. It has high Km value of about 17 mM, resulting in a very low affinity for glucose but a higher selectivity for glucosamine (Km = 0.8 mM) (Navale, 2019; Thorens, 2015). Despite its low affinity for glucose, it has a high glucose capacity to allow for quick glucose equilibrium in the intracellular as well as extracellular compartment that allows it an unlimited contact to

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glucokinase in the hepatocytes (Navale, 2019). It regulates the release of glucose reserves from the liver during fasting and the absorption of glucose by the liver during feeding (Sharari et al., 2020). It also regulates glucose uptake in pancreatic beta cells based on the circulating glucose concentrations. It functions in the kidney for the reabsorbing of filtered glucose in the tubules as well as the absorption of glucose into the blood via the basolateral membrane of the small intestines (Mora & Pessin, 2013).

A defect in the gene encoding for GLUT2 has been associated with a condition called the Fanconi-Bekel Syndrome (FBS) also known as glycogen storage disease XI (Sharari et al., 2020). This is associated with impaired glucose and galactose transport and uptake by hepatocytes, as well as a decrease in insulin release by the pancreas, resulting in hyperglycaemia in the fed state. This condition is also characterized by GLUT2 deficiency in the hepatocytes which impairs glucose export from the hepatocytes into the blood resulting in hypoglycaemia in the fasting state. This diminished glucose stores during the fasting state is aggravated by glycosuria due to the absence of GLUT2 in the proximal tubules to facilitate glucose reabsorption (Sharari et al., 2020).

2.3.3 Glucose Transporter 5 (GLUT5)

GLUT5 is a Class II member of the GLUT-family transcribed by the gene SLC2A5. It has a high affinity (K_m, 6-14 mM) and specificity for fructose and known as the exclusive transporter for fructose (Patel et al., 2015). It is located at the apical surface of the epithelium of the small intestines, the testes, and the proximal tubules of the kidney. It moves fructose from the apical membrane of the small intestines in to the enterocytes after which the fructose is facilitatively transported by GLUT2 from the enterocytes into the blood (Patel et al., 2015). Overexpression of GLUT5 has been linked to conditions such as type 2 diabetes, obesity, breast cancer and pancreatic cancer (Navale, 2019).

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2.3.4 Glucose Transporter 4 (GLUT4)

GLUT4 is one of the 14 members of the GLUT family belonging to Class I of the facilitative transmembrane hexose transporters (Leto & Saltiel, 2012a). It is made up of 12 transmembrane domains and has an N-terminal and a carboxyl group responsible for its responsiveness and membrane trafficking (Vargas, 2022). GLUT4 is a glucose transporter with a strong affinity for glucose located in the cells of the skeletal muscle, fat cells, heart and brain where it is translocated to the plasma membrane of the cells for glucose uptake under the influence of insulin and exercise (Leto & Saltiel, 2012a; Navale, 2019; Patel et al., 2015). In the absence of insulin and exercise up to 90% of GLUT4 is retained in intracellular storage vesicles (Courtney et al., 2021).

The insulin mediated stimulation can occur by two signalling pathways; either through the phosphatidylinositol 3 phosphate (PIP) or the proto-oncoprotein c-Cbl pathway (Hirabara et al., 2012; Vargas, 2022). The PI3P pathway is activated when insulin attaches to an insulin receptor (IR) on a target cell, producing a change in shape in the insulin receptor and subsequent activation of the IR, resulting in the activation of the phosphatidylinositol 2, 3-kinase pathway. With this pathway, phosphatidylinositol 3-kinase (PI3K) phosphorylates phosphatidylinositol 4,5-bisphosphate, which generates an increased intracellular level of phosphatidylinositol 3,4,5-trisphosphate (PIP3). Phosphatidylinositol 3,4,5-trisphosphate activates Akt and 3-phosphoinositide-dependent protein kinase (PDK1), both of which are essential for insulin signalling, such as translocating of GLUT4 from intracellular vesicles to the plasma membrane, allowing for glucose to enter muscle cells (Hirabara et al., 2012; Ijuin & Takenawa, 2012; Leto & Saltiel, 2012a, 2012b).

The c-Cbl proto-oncoprotein is another insulin signalling pathway. The binding of insulin to its receptor recruits associated protein substrates (APS) to the activated insulin receptor,

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causing a dimeric complex of c-Cbl and c-Cbl associated protein (CAP) to migrate into lipid rafts on the cell membrane. Phosphorylation of c-Cbl binds an adaptor protein complex (CrkII) and an exchange factor (C3G) for GTPase TC10. TC10 is found in lipid rafts. Thus, C3G activation of TC10 causes GLUT4 to be translocated to the cell surface, allowing glucose to enter the cells (Baumann et al., 2000; Hirabara et al., 2012; Vargas, 2022).

Although scientific evidence suggests that physical activity plays a role in the movement of GLUT4 from vesicles within the cell to the cell membrane, the exact mechanism involved in translocation has not been well defined. *In vivo* and *in vitro* research indicate that pathways such as AMPK, CaMKII, NOS, and ROS are activated to stimulate the transfer of GLUT4 to the surface of the cell for the entry of glucose (Richter & Hargreaves, 2013; Vargas, 2022).

GLUT4 translocation and expression has been found to be defective in type 2 diabetes patients. A study conducted among type 2 diabetes individuals found normal levels of GLUT4 in their skeletal muscles (Navale & Paranjape, 2016) which may imply a dysfunction or an impairment in the signalling cascade responsible for the translocation of GLUT4 from the cells interior to the surface (Navale & Paranjape, 2016). In rats with MetS there was reduced expression of GLUT4 transport protein in the muscle as compared with healthy rats (Leguisamo et al., 2012b). As a result, GLUT4-related proteins have been identified as possible targets for the treatment of type 2 diabetes. Metformin and thiazolidinediones, for example, have been proven to be effective hypoglycaemic agents due to their action on insulin signalling pathway mediators, which increases GLUT4 trafficking and translocation (Vargas, 2022). When type 2 diabetes patients were not treated, GLUT4 expression was lower than in patients who were treated with oral hypoglycaemic medications (Kampmann et al., 2011). This makes GLUT4 an essential transport protein in glucose homeostasis and metabolism.

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2.4 INSULIN SIGNALLING PATHWAY

Insulin basically functions to maintain glucose homeostasis through its effect on insulin receptors located on target cells in the liver, fat tissue and skeletal muscle. The signalling pathway differs for each of the target tissues as a result of their distinct metabolic effects and roles in glucose homeostasis (Petersen & Shulman, 2018). In the skeletal muscle, insulin functions in facilitating the transport of glucose to the myocytes for its utilization in providing energy through glycolysis and storage through glycogen synthesis. In the liver, it facilitates the uptake of glucose to glycogen synthesis, lipogenic gene expression and inhibits gluconeogenesis. In the adipocytes, insulin causes an increase in the uptake of glucose, and suppresses the breakdown of fatty acids whilst promoting the synthesis of fatty acids and triglycerides (Petersen & Shulman, 2018).

The insulin hormone was discovered in 1921 by Frederick Bantin a Canadian surgeon and Charles Best his Assistant. Their discovery and understanding of insulin and its activity have been a cornerstone in understanding and managing diabetes (Dupont et al., 2022a). Insulin is made up of two polypeptide chains, alpha and beta, linked by two disulphide linkages. It is synthesized in the pancreatic beta cells from preproinsulin, a lengthy polypeptide chain in the beta cells' endoplasmic reticulum (Dupont et al., 2022a). Preproinsulin is further processed by slicing its signal molecule to produce proinsulin, the precursor for insulin production. Proinsulin is moved to the Golgi apparatus and stored in secretory granules within the pancreatic islet cells. Connecting peptides in proinsulin are cleaved by proteases to produce the insulin hormone and subsequently stored in secretory vesicles in the islet cells of the pancreas (Dupont et al., 2022a; Ganea, 1970).

Following the intake of a meal containing carbohydrate or sugars, glucose obtained after digestion is moved from the intestinal lumen to the portal circulation through the action of

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sodium dependent glucose transporter and GLUT2 which elevates the blood glucose concentration. GLUT2 transports glucose to the pancreatic beta cells where it is phosphorylated by glucokinase to release energy in the form of ATP. A rise in ATP in the cytoplasm of the pancreatic beta cells inactivates the ATP sensitive K^+ channels preventing K^+ from leaving the beta cells. This raises the positive charge inside the beta cells, depolarizing the cell membrane and allowing Ca^{2+} to be released into the cell. When Ca^{2+} is present, insulin-filled secretory vesicles are released and dock on the beta cell membrane, where insulin is exocytosed into the general circulation and other metabolic activities occur (Dupont et al., 2022a).

The physiological effect of insulin is achieved by attaching to an Insulin Receptor (INSR), located on the target cells' cell membrane which spans the cell membranes of the targeted cell. It has α and β subunits. The alpha unit is located outside the cell membrane (extracellular) whereas the beta subunit spans the cell membrane, extending into the cytoplasm of the target cells (Dupont et al., 2022a; Petersen & Shulman, 2018).

With an increase blood glucose concentration, the beta cells release insulin from their secretory vesicles. Insulin attaches to the extracellular alpha subunit which activates the intracellular beta subunit through autophosphorylation. This process triggers a cascade of intracellular proteins which facilitates the movement of GLUT4 in the skeletal muscles and adipose tissue and GLUT2 in the hepatocytes to the cell membrane (Petersen & Shulman, 2018; Ryder et al., 2001). The beta subunit located intracellularly is activated through the process of phosphorylation with the addition of phosphate to tyrosine on the β subunit. The activated β subunit in turn activates Insulin Receptor Substrate 1 and 2 (Fig. 2.4) (IRS 1 and IRS 2) through another phosphorylation reaction on tyrosine residues on the insulin receptor substrates. The activated insulin receptor substrates lead to a chain of reactions beginning with tyrosine phosphorylation of PI3K which also activates protein kinase B (Akt) by phosphorylating it on

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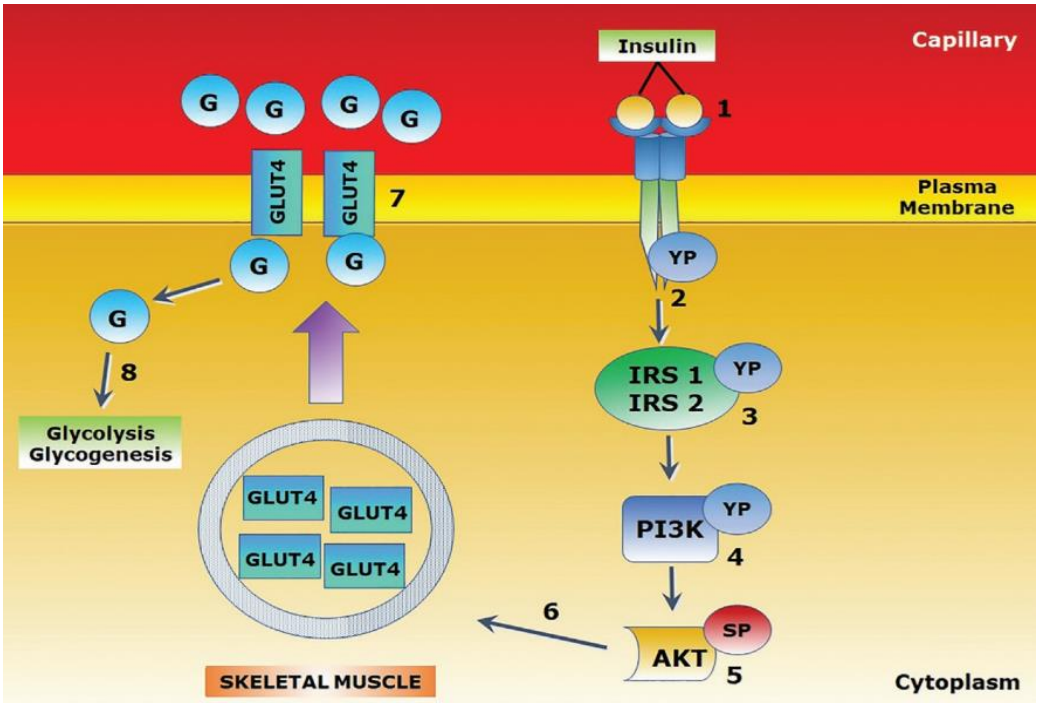

Figure 2.4: Insulin Signalling (Dupont et al., 2022b)**.** Insulin binds to an insulin receptor on target cells which activates IRS1 and IRS2. IRS1 and IRS2 activates PI3K and AKT. This sends a signal to GLUT4 vesicles to move to the plasma membrane. GLUT4 is released from the vesicles and docks to the plasma membrane. Glucose enters the cytoplasm through GLUT4.

serine residues (Fig. 2.4) (Gopalan & Kirk, 2022). Phosphorylation of Akt signals storage vesicles containing GLUT4 to move towards the cells membrane, releasing the GLUT4 transporting proteins which fuses with the cell membrane allowing extracellular glucose into the intracellular domain for storage as glycogen or fat or for energy production (Fig. 2.4) (Gopalan & Kirk, 2022). In summary insulin signalling is characterized by two main steps; activation of the insulin receptor substrates and insulin transduction pathway (phosphorylation of PI3K and Akt) which promotes glucose uptake via GLUT4 (Ryder et al., 2001). Defects in the insulin signal transduction pathway is associated with the pathogenesis of reduced insulin sensitivity; one of the criteria used in diagnosing MetS.

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2.5 THE ROLE OF HEXOKINASE

Monosaccharides from carbohydrate digestion must be transported and phosphorylated before they can be used by the organism. Monosaccharides are transported by glucose transporters (sodium-coupled transporters and glucose transporters), whereas phosphorylation is accomplished by hexokinases. Otto Meyerhof coined the term "hexokinase" (originating from the term hexose and from the Greek word "κινεειν" meaning move), which means "an enzyme that activates glucose, making it ready for fermentation by muscle extracts" (Roberts & Miyamoto, 2015a; van Schattingen, 2021). Phosphorylation of glucose increases the concentration of glucose-6-phosphate intracellularly. This process inhibits the escape of concentrated glucose-6-phosphate from the cells and prepares it for enzymatic action (van Schattingen, 2021). Hexokinases do not only phosphorylate glucose, but also mannose, fructose, glucosamine and 2-deoxyglucose. Hexokinase mediates this process by removing the phosphoryl group located at the terminal end of ATP-Mg and attached it to the oxygen located at the sixth carbon of glucose (van Schattingen, 2021).

There are four isoforms of hexokinase in mammals namely hexokinase (HK) I, II, III and IV. Hexokinase I is mainly located in the brain, red blood cells and also ubiquitously expressed and inhibited by glucose-6-phosphate (van Schattingen, 2021). Hexokinase II is an insulin responsive isoform found in muscles, and adipocytes. Its enzymatic action is influenced by insulin release and concentration. It has the highest Km for glucose and is also inhibited by high concentration of glucose-6-phosphate (G6P) (Roberts & Miyamoto, 2015b; van Schattingen, 2021). Hexokinase III is located majorly in the white blood cells (neutrophils) with a high affinity for glucose and with its activity restricted by increased levels of glucose and G6P. Hexokinase IV is located in the liver, α and β cells of the pancreatic beta cells and hypothalamic neurons. It has a relatively low affinity for glucose compared with HK I-III and regulated by the action of glucokinase regulatory protein in the presence of fructose-6-

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phosphate. Aside glucose it catalyses the phosphorylation of other hexoses although its affinity for the other hexoses is lower than its affinity for glucose hence it often been referred to as "glucokinase" (Msomi, Erukainure, Salau, Olofinsan, & Islam, 2023; Roberts & Miyamoto, 2015a; van Schattingen, 2021).

The release of insulin from secretory vesicles facilitates the release of GLUT4 to the cell surface for glucose passes into cells and phosphorylated intracellularly by HK II. Insulin is responsible for the transcription of HK II gene from which HK II mRNA is transcribed and finally the translation of the HK II protein form the mRNA (Osawa et al., 1996). Reduced levels of HK II in the skeletal muscle and fat cells is observed with type 2 through its effect on insulin sensitivity (Osawa et al., 1996). Resistance to insulin has been reported to reduce HK II expression in obese and type 2 diabetes individuals (Pendergrass et al., 1998). A high fat diet has been observed to down regulate HK II in visceral fat and skeletal muscle resulting in an efflux of non-phosphorylated glucose leading to hyperglycaemia (Pendergrass et al., 1998). This suggests that diet may be a factor in the regulation of HK II. However, there is a paucity of data.

2.6 METABOLIC SYNDROME

The phenomenon of obesity, diabetes, dyslipidaemia and hypertension being present at the same time in an individual was first observed by Crepaldi in 1967 (Blaton et al., 2008). By the 1970s the coining of the term "metabolic syndrome" (MetS) was given by German researchers to refer to the clustering of the symptoms in an individual after which it has since being referred to as either "insulin resistance syndrome", "syndrome X", "plurimetabolic syndrome" and "metabolic syndrome" with metabolic syndrome being the most common term (Blaton et al., 2008). Despite different health organizations coming with varying clinical definition or criteria for classifying or diagnosing the syndrome, some similarities remain.

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From the WHO consultation committee, the initial definition of MetS in 1998, emphasized on insulin resistance as the primary risk factor for MetS: "presence of insulin resistance [(glucose > 6.1 mmol/L (110 mg/dl), 2 h glucose > 7.8 mmol (140 mg/dl)]" plus two additional risk factors such as obesity [(Waist/hip ratio > 0.9 (men) or > 0.85 (women) or BMI > 30 kg/m2)], hypertension (>140/90 mmHg), high triglyceride level (> 1.7 mmol/ (150 mg/dl), reduced high-density lipoprotein cholesterol level less than 0.9 mmol/l in men, and less than 1.0 mmol/l in women, or microalbuminuria (Alberti et al., 2009 ; Blaton et al., 2008); Saklayen, 2018a). The definition included individuals with T2D and the use of the gold standard measure – glucose clamp method to determine insulin resistance (Alberti et al., 2009). Due to the complexity associated with this method it is difficult to use it in the clinical setting and larger epidemiological research (Balkau & Charles, 1999).

However, in 1999 The European Group for the Study of Insulin Resistance (EGIR) criticized the definition by the WHO 1998 Expert Committee (Balkau & Charles, 1999). They recommended that, because the syndrome included non-metabolic features, "insulin resistance syndrome" will be a more appropriate name (Balkau & Charles, 1999). They also critiqued the criteria for diagnosing the syndrome, requiring reduced glucose tolerance or resistance to insulin, as well as a combination of any of the other risk factors. Their proposed definition was for non-diabetic individuals. They proposed that before a diagnosis can be made, all the following assessments should be measured: "high fasting insulin levels (greater than the 75th percentile), hyperglycaemia (≥ 6.1 mml/l); hypertension (systolic/diastolic blood pressures ≥ 140/90 mmHg or treated for hypertension); dyslipidaemia (triglycerides >2.0 mmol/l or HDL-Cholesterol <1.0 mmol/l or treated for dyslipidaemia); central obesity (waist circumference ≥ 94 cm in men and ≥ 80 cm in women)" (Balkau & Charles, 1999; Grundy et al., 2005;. Huang, 2009). They recommend insulin at fasting levels as a better proxy for measuring insulin resistance than the clamp method recommended by the WHO expert committee in their

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definition (Balkau & Charles, 1999). When comparing the EGIR criteria with that of the WHO, they focused more on abdominal obesity and excluded people who had already been diagnosed with type 2 diabetes given that the resistance to insulin is recognised as a significant risk indicator in type 2 diabetes and because measuring insulin resistance in people with type 2 diabetes was complex. It also disregarded microalbuminuria as a diagnostic criterion for metabolic syndrome (Balkau & Charles, 1999; Grundy et al., 2005).

In 2001, another criterion for diagnosis was introduced by "The National Cholesterol Education Program (NCEP) Adult Treatment Panel III" (APT III). They introduced other markers that can be useful in diagnosing metabolic syndrome with the main goal of easily identifying individuals with an increased long-term risk of arteriosclerotic cardiovascular disease (ASCVD) and put in intervention to reduce the risk (Grundy et al., 2005). In contrast to the WHO and EGIR definitions, ATP III requires a diagnosis of at least three of the five risk factors rather than the measurement of insulin resistance or the presence of a single component (Huang, 2009). NCEP-ATP III defined metabolic syndrome "as the presence of three or more of the following 5 risk factors; increasing waist circumference (> 102 cm for men and > 89 cm in women), high blood pressure (> 130/85 mmHg), high fasting triglyceride (TG > 8.3 mmol/l/ 150 mg/dl), low high density lipoprotein cholesterol (HDL-C) (< 2.2 mmol/l or 40 mg/dl for men and <2.8 mmol/l or 50 mg/dl in women) and fasting blood glucose over 100 mg/dl or 5.6 mmol/l" (Blaton et al., 2008). In summary, the definition of metabolic syndrome according to NCEP-ATP III includes high blood sugar levels or resistance to insulin, hypertension, "atherogenic dyslipidaemia", and central obesity (Alberti et al., 2009; Blaton et al., 2008) using readily available laboratory tests and measurements (P. L. Huang, 2009).

The International Diabetes Federation (IDF) and the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) sought to harmonise the differences between

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the various definitions of metabolic syndrome in 2005. They agreed on four risk factors (hyperglycaemia, elevated triglyceride level, low HDL levels and elevated blood pressure) with disagreement with the abdominal obesity (Alberti et al., 2009). The IDF did not agree with insulin resistance being a requirement for diagnosis but rather abdominal obesity as the necessary factor using waist circumference instead of waist-to-hip ratio. Also, they did not reach a consensus on the cut off for waist circumference between the two organisations (Alberti et al., 2009; Huang, 2009). The IDF defined "abdominal obesity in people of European origin (Europids) as ≥94 cm for men and ≥80 cm for women"; whereas the "AHA/NHLBI recommended cut-off points of ≥102 and ≥88 cm, respectively, for males and females". The AHA/NHLBI cut-off values are equivalent to a BMI classification of obesity, whereas the IDF cut-off is equivalent to a BMI of 25 kg/m2 in males. Furthermore, for a more accurate assessment of health risks, IDF recommends ethnic-specific cutoff limits for waist circumference (Alberti et al., 2009).

A joint discussion held between IDF and AHA/NHLBI came to a consensus with the remaining differences in defining metabolic syndrome. It was agreed that abdominal obesity should not be the main factor in defining MetS but should be one of the 5 criteria needed to make a diagnosis. Metabolic Syndrome was thus diagnosed as the co-existence of three of the five risk factors: higher abdominal or waist circumference (with different cut-offs for various populations and countries), elevated TG (150 mg/dl/1.7 mmol/l), reduced HDL-C (40 mg/dl/1mmol/l in males, 50 mg/dl/1.3mmol/l in females), elevated blood pressure (130/85 mmHg), and raised fasting blood sugar (100 mg/dl) (Alberti et al., 2009). In summary based on all the major organization, it has been agreed that no one risk factor should be a required component in defining metabolic syndrome however waist circumference will remain a useful tool in assessing risk of metabolic syndrome. Additionally, the presence of any three of the five risk variables outlined will identify an individual as having metabolic syndrome (Alberti et al.,

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2009). Thus, WHO and IDF definition are centred on glucose indicators and obesity whereas the AHA/NHLBI is based on assessing cardiovascular disease risk (Noubiap et al., 2022a).

The prevalence of metabolic syndrome has been shown to increase alongside the increase obesity and type 2 diabetes pandemic which are both moderators in the development of metabolic syndrome (Saklayen, 2018b). The prevalence of metabolic syndrome does not only vary across different population but also based on the criteria used (Noubiap et al., 2022a). The first meta-analysis conducted to assess the global prevalence of metabolic syndrome among adults revealed a prevalence ranging from 12.5% to 31.4% based on the diagnostic criteria (Noubiap et al., 2022a). Based on the WHO, EGIR, ATP-III and AHA/NHLBI the prevalence for MetS was 20.9%, 16.0%, 25.4% and 29.1% respectively. The highest prevalence of 31.4 % was reported using the Joint Interim Statement (JIS). In this same metanalysis, the prevalence was also estimated for each WHO region. Even across the different MetS definition, the Americas (18.9% (AHA/NHLBI) - 46.4% (JIS)) and Eastern Mediterranean (30.5% (AHA/NHLBI)-35.9% (JIS)) WHO regions had the highest prevalence. Seventy-eight (78) prevalence studies was pulled for the WHO Africa region with a prevalence ranging from 18.9% (AHA/NHLBI) to 31% (JIS) (Noubiap et al., 2022a). The prevalence of metabolic syndrome was analysed based on the country's level of income. Lower middle-income countries (12.9% to 28.4%) had the lowest MetS prevalence with the highest prevalence observed in upper middle income (24.4% to 34.1%) and high-income countries (22.5% to 30.6%). Based on the MetS definition, the highest prevalence was recorded for the JIS criteria for MetS. There was no substantial link between the income level of a nation and MetS, implying that physical inactivity and a poor diet are the main culprits of MetS in all countries, regardless of wealth. (Noubiap et al., 2022a).

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Saklayen (2018a), reported that about a third of the population in the US have MetS with South Asian Americans having a higher prevalence (Palaniappan et al., 2011). The prevalence of the predicted metabolic syndrome in Asian women was higher (30%) compared to the non-Hispanic whites (12%). Similarly, among the men, the non-Hispanic whites had a predicted prevalence of 22% compared with the 43% in the Asians (Palaniappan et al., 2011). According to (Li, Qiu, Ma, & Geng, 2023) between 1999 and 2014, metabolic syndrome in the US rose from 27.6% to 32.3% however there was a decline in the mortality rate and cardio-cerebrovascular death during the period. Contrary to the finding of (Saklayen, 2018a), women had an increased risk of being diagnosed with MetS but mortality was higher in the men than the women. High waist circumference, low HDL-C and high fasting plasma glucose contributed mostly to MetS in the US population (W. Li et al., 2023). The NHANES data from 2011 to 2016, indicated a prevalence of 34.3% for MetS in US based on the NCEP/ATP III criterion. Surprisingly, there was no variation in prevalence between males and females, however, in terms of ethnicity non-Hispanic whites had the lowest prevalence compared with the Hispanics and other race (Vaduganathan et al., 2020).

A prevalence of 14% was found in Europe and North America, with a 2-fold and 4.9-fold rise in the number of new cases of Arteriosclerotic Cardiovascular Disease and mortality, respectively (Paquette et al., 2023). A prevalence of 20.6% was recorded among Malaysian public university employees, with men having a higher prevalence than women. Diabetes, BMI, and dyslipidaemia were associated with the development of MetS in women, while type 2 diabetes and hypertension were associated with MetS in men (Manaf et al., 2021). MetS syndrome was diagnosed by Manaf et al. (2021) using the Joint Interim Statement (JIS). In the United Arab Emirates, a prevalence of 37.4% was reported with a higher prevalence in female Emirati and Asian men according to the NCEP-ATP III definition (Mahmoud & Sulaiman, 2022). Among children and adolescents, a prevalence of 3% and 5% was reported in children

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and adolescents respectively with variations in prevalence observed across countries regions (Noubiap et al., 2022b).

Metabolic syndrome is due to a combination of sedentary lifestyle, excessive caloric intake and genetic predisposition (Panagiotis, 2022). Evidence from cohort, cross sectional, ecological and randomized control trial have suggested a link between excessive intake of refined sugars on cardiovascular and metabolic health (obesity, diabetes and insulin resistance) (Macdonald, 2016a; Panagiotis, 2022). The high prevalence of MetS and its associated increased morbidity and mortality risks reported across studies highlights the urgency for lifestyle modifications to curb this menace. The subsequent section will be reviewing the various indicators for metabolic syndrome and their relationship with the intake of both nutritive and low/no calorie sweeteners.

2.6.1 Insulin Resistance

After eating a carbohydrate rich meal, in response to the high blood glucose as previously described, insulin is released to assist in glucose uptake by the hepatocytes, muscles and fat cells. In addition to this it facilitates glycogen storage and fatty acid production. When the blood glucose reduces, insulin secretion is inhibited or reduced to permit counter regulatory hormones such as glucagon to be released for the release of glucose form glycogen stores and breakdown of fatty acids from fat stores (Guo, 2014).

Insulin resistance is a medical condition whereby muscles cells, fat cells and hepatocytes are not responsive to insulin's signalling hence disrupting the movement of glucose from the blood into body cells. This defect in insulin signalling can occur either through the desensitization of the Insulin Receptor, suppression of its Receptor Proteins or the inhibition of PI3K. A defect in either of these signalling pathways will interfere with moving GLUT4 containing vesicles to the surface of the cell and a decrease in the rate or glucose entry into cells of target tissues (Guo, 2014).

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Insulin resistance mostly begins in the muscle which accounts for 70% of the glucose removal from the blood. In response to the chronically elevated levels of glucose in the blood, the beta cells continue to produce more insulin, which eventually exhausts them and causes them to die ; (Freeman & Pennings, 2021b; Santoleri & Titchenell, 2019). Impaired glucose uptake by the muscles makes excess glucose available for uptake by the liver facilitating *de novo* fatty acid synthesis and production of free fatty acids which is stored in other tissues aside the adipose tissue (ectopic fat deposition). Ectopic fat is commonly stored in the heart, muscles, liver, and abdominal region. Accumulation of ectopic fat inside the cell leads to phosphorylation of IRS1 on serine amino acid instead of tyrosine residues. Serine-phosphorylated IRS suppresses the receptor substrates instead of activating it, hence are not able to activate PI3K which culminates in failure to signal GLUT4 containing vesicles to the surface of the cell for glucose entry in to the cytosol (Snel et al., 2012).

The hyperinsulinaemic euglycemic pump (HEC) is the benchmark for assessing insulin resistance (Gastaldelli, 2022; Gutch et al., 2015). It involves infusing insulin at a physiological rate while also infusing glucose to keep the plasma glucose level constant. The rate at which glucose appears in the blood should equal the amount of time it takes for glucose to be cleared from the blood till the plasma level achieves physiological concentration (Gastaldelli, 2022). Due to its relatively expensive and time demanding nature, other indices such as Homeostasis Model Assessment Insulin Resistance (HOMA-IR), Matsuda Index and Quantitative Insulin Sensitivity Check Index (QUICKI) have been suggested for use in clinical research (Freeman & Pennings, 2021b; Gastaldelli, 2022; Gutch et al., 2015). In clinical practice, measurement of insulin resistance has not been incorporated, Instead, alternate surrogate such as TG/HDL ratio, LDL concentration, triglyceride levels, cholesterol, BMI and waist-to-hip ratio are used in assessing an individual's risk in developing insulin resistance (Freeman & Pennings, 2021b; Gastaldelli, 2022).

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Mathews et al was the first to develop HOMA-IR in 1985 which was later updated in 1988. The HOMA index is effective in assessing the body's sensitivity to insulin. It is founded on the premise that blood glucose and insulin concentration are a function of beta cell adequacy with an assumption that a 35-year-old normal weight adult should have a fully functioning β-cell (100%) with an insulin resistance of 1. It has been shown to be an effective epidemiological and clinical measurement for insulin resistance (Gastaldelli, 2022). HOMA-IR provides the insulin-glucose homeostasis by a mathematically derived equation which is a product of the fasting plasma glucose and fasting plasma insulin "(Insulin (mU/L) × glucose (mmol/L)]/22.5)" (Ghosh & Collier, 2012). HOMA β-cell function can also be measured to access the beta cell function "[20 × insulin mU/L]/[glucose (mmol/L) − 3.5]" (Ghosh & Collier, 2012).

Although HOMA has been identified as a robust technique in measuring IR, there is no single threshold or cutoff for HOMA-IR. It differs across population or ethnicity, underlying disease condition, age and gender (Harbuwono et al., 2023). Studies conducted in different geographical locations used the percentile criterion as cuts offs with the criterion ranging from ≥1.7 to ≥3.8 (Hedblad, Nilsson, Janzon, & Berglund, 2000; Ascaso et al., 2001; Do, Lohsoonthorn, Jiamjarasrangsi, Lertmaharit, & Williams, 2010; Esteghamati et al., 2009; Gayoso-Diz et al., 2013b; Geloneze, Repetto, Geloneze, Tambascia, & Ermetice, 2006; Hedblad et al., 2000; Marques-Vidal et al., 2002; Nakai et al., 2002; Sumner & Cowie, 2008; Tomé et al., 2009). In validating the HOMA-IR against the HEC, a threshold of >3.22 was obtained for pubertal and >2.91 for post pubertal (Silva et al., 2023a). The HOMA-IR threshold for predicting MetS in a healthy Chinese population aged 45 years was 1.7 and 1.78 in men and women respectively (Lin et al., 2022). Despite the variations in HOMA-IR, this threshold has been recommended in assessing risk; >4.65 or >3.65 (if BMI >27.5) or 2 in the presence of Non-Alcoholic Fatty liver disease (Gastaldelli, 2022).

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The QUICKI method in assessing IR is very similar to the HOMA model however it measures insulin sensitivity as opposed to resistance. The underlying difference is that the QUICKI method log transforms the glucose insulin product [1/ ($\log_{10}G_{0mg/dl}$ + $\log_{10}I_{0mu/ml}$). The criteria for the QUICKI and revised QUICKI is <0.33 and <0.37 respectively (Gastaldelli, 2022).

Matsuda and Defronzo created the Matsuda Index from fasting plasma glucose and insulin levels as well as an oral glucose tolerance test (OGTT). It is calculated using the formular = $1000\sqrt{G_0I_0G_{mean}I_{mean}}$ where G_0I_0 represent the fasting glucose and insulin level respectively and $G_{mean}I_{mean}$; the mean glucose and insulin concentration during OGTT (Gastaldelli, 2022; Gutch et al., 2015).

Abnormal lipid metabolism has been linked to a higher likelihood of developing diabetes, MetS, and, consequently, CVDs (Wang et al., 2023). The TG/HDL ratio measures the relationship between triglyceride level and high-density lipoprotein. Triglycerides (TG) are the type of fat used mainly for energy in the body. High-density lipoprotein, on the other hand, is known as the "good fat" or "good cholesterol" because it removes bad cholesterol from the bloodstream to the liver for excretion. High triglyceride level is a major concern, but high HDL-C levels are protective against metabolic disorders (CDC, 2023). Clinical investigations have shown that people with high TG levels had low HDL-C levels (Wilson & Grundy, 2003). The joint effect of TG and HDL which is the triglyceride to HDL-C ratio is seen as a useful predictor to insulin resistance compared to triglyceride or HDL-C (Chiang et al., 2011). Despite its usefulness in assessing the risk of Insulin resistance, there is no optimal threshold due to factors such as ethnicity, gender and existing comorbidities (Gong et al., 2021).

In summary, HEC is the gold standard for measuring IR, however due to the cost and complexity of the test, other surrogate measures such HOMA-IR, QUICKI, Matsuda, TG/HDL-C ratio and BMI are being used in clinical research and epidemiological studies.

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Because of differences in ethnicity, gender, and individual risk factors, it is recommended to utilise each measure in conjunction with others.

2.6.1.1 Insulin Resistance and the Intake of Sweeteners

In the 1970's, sucrose was viewed as the main culprit for diabetes, CVD and other chronic conditions as published by Yudkin (1972a) in his book "Pure white and deadly". However increasing research from epidemiological and experimental studies have extended these health effects to include intake of fructose, fructose corn syrup and glucose (Macdonald, 2016a).

As discussed previously, the main end products in the digestion of nutritive sweeteners are glucose and fructose. Excessive intake of fructose beyond the absorptive capacity of the small intestines may end up in the colon where intestinal bacteria mostly referred to as microbiomes act on them to produce glucose, glycerol, organic acids, uric acids, short chain fatty acids. The metabolites are transported back to the liver which serves as substrates for *de novo* synthesis of fatty acids and triglycerides in the liver. Also, fructose intake beyond physiological levels can promote *de novo* lipogenesis in the hepatocytes (Yu et al., 2021). This can result in lipid deposition in the liver and insulin insensitivity in the hepatocytes (Macdonald, 2016a; Yu et al., 2021). Uric acid as a by-product of fructose metabolism can induce insulin resistance through its effects in fatty tissues (Johnson et al., 2013).

A meta-analysis conducted in the United States identified sugary beverage consumption as an independent risk factor for the development of MetS and T2D (Malik et al., 2010). In an econometric analysis, the duration and degree of sugar intake had a significant association with the prevalence of diabetes mellitus in a dose-dependent manner and a decrease in sugar intake was significantly correlated with a decrease in T2D prevalence independent of other factors such as socioeconomic, changes in dietary pattern, physical activity, and being

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overweight (Basu et al., 2013). In this analysis, no other food group or type was individually associated with diabetes after controlling for obesity and other confounders (Basu et al., 2013).

Sánchez-Lozada et al. (2010a) in an animal model of 2 intervention groups, gave one group combination of fructose and glucose (FG) and another group free sucrose (S). Both sugars administered were high dosages. At the end of the study, both intervention groups had induced hepatic alterations with a triglyceride accumulation in the liver, increased level of inflammatory markers and insulin resistance. Mice fed a 38.5% sucrose diet had lower glucose tolerance, as well as lower hepatic Glucokinase activity and impaired GLP-1 release (Sakamoto et al., 2012). Hepatic glucokinase is a key enzyme involved in glucose metabolism in the liver. Its activity has been found to be reduced in those with type 2 diabetes and insulin resistance (Jiang et al., 2008).

After 42 days of administering 15% white sugar and brown sugar, body weight, insulin resistance, and brain-derived neurotrophic factor all increased. These changes, however, were more pronounced in rats given white sugar as opposed to brown sugar (Shamsi-Goushki et al., 2021a). Leibowitz et al. (2018a) in their study compared the effects of a diet high in fructose and saccharin on blood glucose levels and insulin resistance after 7 weeks of treatment. Both treatment groups had a markedly increased fasting blood glucose. However hepatic insulin was significantly raised in the high fructose group (3.4±0.4 ug/L) compared with the control (2.3±0.36 ug/L) and saccharin (2.1±0.3 ug/L) administered groups. Rats fed with high fructose diet developed fatty liver but not in the control or saccharin group.

Figlewicz et al. (2009a) conducted an experimental study using rats with three different treatment groups administered with moderate concentrations of sweeteners over a period of 10 weeks; high fructose corn syrup, high fructose corn syrup with an appetite suppressant and the stevia group. At the end of the study, there was no evidence of liver steatosis and glucose

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intolerance. This is in discordance with finding by Leibowitz et al. (2018a) who reported marked changes in the liver and fasting glucose. This could be because in the latter study, the rats were administered with the sweeteners 3x/week and only at night, thus reducing the duration of exposure to the sweeteners.

Also Leibowitz et al. (2018b) administered high doses of the sweeteners as compared with the moderate dosages administered by (Figlewicz et al., 2009). Valle et al. (2020a) induced obesity in rats and compared the effect of chronic low intake of sucrose, natural sweeteners (maple syrup, corn syrup, agave syrup, molasses, brown rice syrup, honey) fructose and sucrose. The control group was fed with normal rat chow and sucrose for 8 weeks. Sucrose consumption was associated with insulin resistance compared to the natural sweeteners and fructose. However, all the sweeteners induced hepatic steatosis. Findings of this suggested that in the presence of obesity, natural sweeteners may be less harmful alternatives to sucrose.

The same authors evaluated metabolic responses to the intake of natural sweeteners in healthy rats in a previous study. Rats were gavaged with high doses of natural caloric sweeteners (pure maple syrup, molasses, organic brown rice syrup, organic blue agave syrup, golden corn syrup and pure natural honey). Maple syrup elicited a better metabolic response compared with the other natural sweeteners. Rats gavaged with maple syrup had lower glucose and insulin levels. On the other hand, honey caused a spike in insulin and increased release of amylin and gastric inhibitory peptide (GIP) which has effects on β-cells and insulin resistance (St-Pierre et al., 2014). Bigos et al. (2012a) administered Sprague Dawley rats with sucralose, sucrose, and maltodextrin for three weeks at moderate doses (10%). Fasting HOMA-IR showed no significant difference among the groups however the group administered with sucralose had lower postprandial insulin and glucose concentration. The lack of a significant difference in

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the HOMA-IR could be due to the short duration of administration in addition to moderate concentration of the sweeteners.

Administration of stevia preparations at a concentration of 250-500 mg/kg/day effectively lowered fasting blood glucose and HOMA-IR at the end of the research in diabetic-induced Wistar rats. A histological analysis also showed no increase in the pancreatic beta cells (Akbarzadeh et al., 2015). Similar results were obtained by Ahmad & Ahmad (2018a) after administering different doses (200, 300, 400 and 500 ppm/kg b.w) of stevia extract to diabetic rats after 8 weeks. Both random and fasting blood glucose, glycated haemoglobin, insulin level and liver glycogen reduced significantly in the intervention group in comparison with the diabetic controls and non-diabetic controls. Although (Moroni et al., 2016) also reported an improvement in glucose tolerance after 2 months intraperitoneal administration of stevia extract, interestingly insulin levels increased coupled with insulin resistance. At cellular level, the islets of Langerhans were hyperplastic also indicating an over production of insulin which could lead to the beta cells over-working. Anti-glycaemic property of stevia extract has been associated with the presence of stevioside. A clinical trial in forty-eight diabetes patients revealed an increase in glucose level and glycated haemoglobin after 8 weeks administration of honey (Bahrami et al., 2009). There was a reduction in glycated haemoglobin after 8 weeks of administration with 10% honey in Sprague Dawley rats (Bahrami et al., 2009), conversely long-term administration (52 weeks) of 10% honey to Sprague Dawley rats showed a marked reduction in glycated haemoglobin (Chepulis & Starkey, 2008a).

The evidence on the effect of the intake of sweeteners on insulin resistance and blood glucose levels remains controversial, even with the intake of the proposed healthy options like honey and stevia extracts. Across the studies, there is a lack of uniformity in dosage calculation

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making comparison of results challenging. The duration of intake, dosage and type of sweetener may account for some of the findings.

2.6.1.2 Sweeteners and GLUT4

As discussed in previous sections, metabolic syndrome is characterized by insulin resistance. GLUT4 is the primary insulin-responsive glucose transporter is responsible in maintaining postprandial glucose concentration and also involved in insulin signalling and in insulin responsive cells. In an animal model of metabolic syndrome, it was demonstrated that GLUT4 decreased alongside all the characteristics of MetS compared to rats without metabolic syndrome (Leguisamo et al., 2012a). Insulin resistance was improved in rats through increased expression of GLUT4 by feeding them with l-carnitine and omega 3 (Zayed et al., 2018). Thus, the relationship between insulin resistance and GLUT4 expression emphasizes the role GLUT4 plays in insulin signalling; upregulation of GLUT4 expression improves glucose homeostasis and the body's responsiveness to insulin whereas its reduction reduces insulin stimulated intake of glucose into cells (Chadt & Al-Hasani, 2020). Treatment with Stevia Rebaudiana Bertoni extracts increased glycemia in diabetic-caused rats by raising the expression of GLUT4 in skeletal muscles and the kidneys (Bayat et al., 2020).

Excessive intake of sweeteners (caloric and non-caloric) has been found to cause insulin resistance through excessive weight gain, fat deposition in hepatic and extra hepatic tissues. In the gastrocnemius muscle, high fructose corn syrup impaired insulin signalling pathway by reducing the expression and movement of GLUT4 to the cell membrane (Benetti et al., 2013). Studies on the development of insulin resistance have focused on fructose corn syrup or individual sugars. There is little research on the effects of granulated white sugar, brown sugar, and processed stevia sweetener on insulin resistance. Furthermore, the molecular mechanism

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behind the adverse effects of specific sweeteners on insulin resistance, such as GLUT4 expression, has not been investigated.

2.6.2 Dyslipidaemia

Dyslipidaemia is defined as an imbalance in blood lipid profile, which includes total cholesterol, TG, HDL-C and LDL-C (Pappan & Rehman, 2022). Triglycerides also known as triacylglycerol (TAG) is the major dietary fat found in plant and animal fat and fat stores in humans. It comprises three molecules of fatty acids linked to a glycerol backbone (Mansbach, 2004). Digestion of TAG mainly begins in the duodenum where it is emulsified by bile salts and hydrolysed through the action of pancreatic lipase to produce free fatty acids and cholesterol (Mansbach, 2004). The end-product of TAG digestion is packaged into chylomicron and transported to the lymphatic and bloodstream. Once in the bloodstream they move to the liver where it is stored as fat or moves to the fat cells to be stored (Mansbach, 2004).

Triacylglycerol (triglyceride) and cholesterol are insoluble in water and hence transported in the blood with the help of lipoproteins. Lipoproteins are round particles made up of cholesterol esters, TAG, phospholipids, free cholesterol, and apolipoprotein. Lipoproteins are classified into seven types based on their size, lipid makeup, and apolipoprotein; these include chylomicrons, chylomicron remnants, very low-density lipoprotein, intermediate-density lipoprotein, low-density lipoprotein, HDL-C, and lipoprotein (Feingold & Grunfeld, 2000; Valle et al., 2020b). Based on atherogenicity, lipoproteins are divided into pro-atherogenic lipoprotein (chylomicron remnants, VLDL, IDL, LDL and Lp) and anti-atherogenic lipoproteins (HDL) (Feingold & Grunfeld, 2000). For the purpose of this study, this review will focus on the following lipoproteins, LDL, VLDL and HDL.

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Very Low-Density Lipoprotein is a triglyceride rich lipoprotein made up in the liver (Lee, Akhmedov, & Chen, 2022b). It has a very high fat content (90%) and low protein content (10%) hence its very low density. Seventy percent of its fat mass is made up of cholesterol ester and fatty acid. Its main core protein is apolipoprotein (apo) B100, and it also contains other proteins including apoCI, apoCII, apoCIII, and apoE (Lee et al., 2022b; Casso & Farzam, 2022). Cholesterol, TAG, and proteins are moved to peripheral cells by the VLDL. Triglycerides in VLDL are metabolized in the cells of the muscle and adipose tissue by lipoprotein lipase to produce free fatty acids and IDL which serves as precursor for LDL production and stored in the liver (Feingold & Grunfeld, 2000). One of the main risk factors in the development of atherosclerosis is high VLDL concentration (Zhao et al., 2017). Excess intake of sugars mean high amount of fructose and glucose are made available for absorption and converted to glycerol-3-phosphate an intermediate product for the manufacturing of free fatty acids and triglyceride and package into VLDL and secreted into the circulatory system (Fried & Rao, 2003).

Low density lipoprotein is the most abundant pro-atherogenic lipoprotein in the body. It is derived from IDL and VLDL and further enriched with cholesterol hence being the major cholesterol carrier in the blood. LDL comes in varying sizes and densities. The small dense LDL have been shown to be more pro-atherogenic because they have a low affinity for LDL receptors causing it to be retained for a long period in the circulation. They are also able to easily penetrate the walls of the blood vessels which gets them trapped in the arterial wall where they are easily oxidized aided by trapping macrophages. This process is observed in the formation of atherosclerosis (Ahmed et al., 2022; Casso & Farzam, 2022; Feingold & Grunfeld, 2000).

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High density lipoprotein, on the other hand, plays the opposite role in cholesterol transport, removing and transferring cholesterol from peripheral tissues and the circulation back to the liver, where it is eliminated from the body via bile. This physiological role by HDL makes it anti-atherogenic and commonly termed as the "good cholesterol" (Casso & Farzam, 2022; Feingold & Grunfeld, 2000). Unlike LDL, IDL or VLDL, high density lipoprotein has a high protein content (about 50%) and rich in phospholipids making it denser (Schaefer et al., 2009).

Clinically, a lipid profile test is done to assess the level of the different types of lipids in the blood which gives an indicator of an individual's risk of metabolic syndrome or CVD. An increase in pro-atherogenic lipid levels is related to an increased cardiovascular risk, whereas an increase in HDL levels is protective against atherosclerotic heart disease (Gasevic, Frohlich, Mancini, & Lear, 2014a; Ahmed et al., 2022). Scientific evidence suggests that, lipid ratios are better in predicting cardiovascular disease risk and metabolic syndrome as opposed to individual lipid levels and helps to alleviate misclassification of individuals with an increased risk (Gasevic et al., 2014a; Kałużna et al., 2022). Also, sometimes LDL and other lipids could be normal whereas there may be elevation in other atherogenic lipoproteins hence the need for the lipid ratios being widely used (Kałużna et al., 2022).

Several lipid ratios or atherogenic indices have been proposed to improve the prediction capacity of the lipid profile test. These ratios are good metabolic indicators, presenting a more precise picture when regular clinical indicators are unable to assess danger, and their predictive value is greater than that of their individual values (Millán et al., 2009). The lipid ratios that will be discussed in this review are: Castelli Risk Index I (CRI-I) and II (CRI-II), Atherogenic Co-efficient (ACoE), and Atherogenic Index of Plasma (AIP). These lipid ratios demonstrate the link between pro-atherogenic lipids and lipoproteins (total cholesterol (TC), TG, LDL-C) and anti-atherogenic lipids (HDL-C).

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Coronary Risk Index-I is a ratio of TC to HDL-C (TC/HDL-C), which are both independent risk variables for coronary artery disease development. CRI-II is a similar ratio of LDL-C to HDL-C. Using data from the Framingham and Coronary Primary Prevention Trial Studies, four indicators were examined to determine their effectiveness in predicting coronary heart disease. When compared to total cholesterol or HDL-C alone, CRI-I was a stronger predictor of coronary heart disease (CHD) (Kinosian et al., 1994). Despite its usefulness, it has been demonstrated that the Atherogenic Index of Plasma is more reliable in predicting myocardial infarction (Gaziano et al., 1997), insulin resistance (McLaughlin et al., 2005) and metabolic syndrome (Cordero et al., 2008; Wakabayashi & Daimon, 2019).

In a cross-sectional study of participants from Aboriginal, Chinese, European and South Asian origin, AIP was seen as a better marker in identifying metabolic syndrome in both sexes in comparison with CRI-I, CRI-II and ACoE (Gasevic et al., 2014b). Among the Chinese population AIP was good indicator of MetS based on the JIS and NCEP/ATP III (Chen et al., 2014). Insulin resistance can result in increased production of fatty acids and subsequently increased triglycerides coupled with a reduction in HDL (Moriyama, 2020). Several cut off points have been suggested for the various lipid ratios depending on ethnicity, gender and other underlying disease conditions (Ford, Meng, Mokdad, & Reaven, 2008). In Spain, for instance, the suggested AIP cut-off number is 2.75 in men and 1.65 in women (Cordero et al., 2008) whereas in an Iranian population of people living with diabetes, the proposed cut-off for AIP was 4.7 in men and 3.7 in women (Hadaegh et al., 2010).

2.6.2.1 Intake of sweeteners and dyslipidaemia

Sugars are primary source of energy in humans. There is an established link between the metabolism of glucose, fructose, fatty acids and *de novo* lipogenesis (Chen et al., 2019) with evidence showing that replacing dietary fat with carbohydrate can result in an elevation of

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triglyceride and a reduction in HDL-C, however this was influenced by the type of carbohydrate being consumed (Johnson et al., 2009). Sugar sweetened beverages and added sugars (caloric and non-caloric) have been associated with an imbalance in the lipid profile (Haslam et al., 2022; Welsh et al., 2010a). The end product of caloric sweeteners are mainly glucose and fructose with about 25% of the fructose converted to glucose. Excess intake of sugar means an increase in the quantity of substrates entering the glycolytic pathway and lipogenesis. Metabolism of glucose and fructose has been discussed extensively in section 2.3 of this review.

Based on the Framingham Offspring Study (FOS) and the Women's Health Study, sugar sweetened beverage was associated with an increase in LDL-C, non-HDL-C, TG, apoB, rich lipoprotein particles and LDL particles and a decrease in HDL-C and apoA1 (Haslam et al., 2022). In assessing the relationship between the intake of caloric sweeteners and dyslipidaemia among US adults, increasing intake of caloric sweeteners positively associated with TG and LDL-C and a reduction in HDL-C (Welsh et al., 2010a). Excess intake of energy from sugar sweetened beverages and caloric sweeteners is converted to lipids (Wei et al., 2022). Lipid deposition in the liver by different types of sugar was assessed using a cell culture experiment. Fructose and sucrose were observed to induce hepatic steatosis (Wei et al., 2022). When the production of triglycerides, exceeds the ability of transport proteins to transport them, there is a deposition of fat, which is similar to what happens with fatty liver (Wei et al., 2022).

Wistar rats were treated with three different doses of honey (0.04, 0.09, 0.13 mg/ml) and white sugar (0.02,0.03, 0.04 mg/ml) for 4 weeks. Low dose (0.02 mg/ml) of white sugar caused a decrease in LDL, TC and TG with a significant decrease in HDL-C with medium-dose of white sugar. All doses of honey were observed to significantly decrease TC, TG and LDL with medium-dose honey significantly increasing HDL-C. Also, in comparing medium-dose of the

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two sweeteners, honey significantly reduced Castelli Risk Index I (CRI-I) (Nwachuku et al., 2017). In complementing the hypolipidemic effect of honey, administering a dose of 50 mg/kg of honey in diabetic rats resulted in a significant decrease in TC, TG and VLDL-C after 3 days of administration (Öztaşan et al., 2005).

In another animal experimentation model, different dosages of honey (1000, 2000, 3000 mg/kg) were administered to alloxan induced diabetic rats. Honey had positive effect on TG, VLDL, HDL, CRI-I, CRI-II and Atherogenic index with no significant reduction in LDL-C confirming the hypolipidemic effect of honey (Erejuwa et al., 2016a). They suggested that this effect observed in honey may be due to its protective effect on the pancreas, protecting the pancreatic beta cells from oxidative damage which will enable the β cells to work efficiently to produce adequate amount of insulin. Although honey is rich in simple sugars, it also possesses anti-inflammatory, anti-oxidant and anti-bacterial properties which may be contributing to its lipid lowering properties (Gholami et al., 2022).

Despite these positive findings, a meta-analysis in human clinical trials revealed that honey had no significant effect on lipid markers (total cholesterol, triglycerides, LDL-C, HDL-C, and LDL-to-HDL ratio) (Gholami et al., 2022). This lack of observed relationship could be a result of the significant heterogeneity due to differences in terms of dietary regimens, doses, population, and type of honey being used. Also, most studies were conducted in Eastern countries which limits the generalizability of the meta-analysis. Another systematic review on the lipid lowering effect of honey showed a positive association on both pro-atherogenic and anti-atherogenic lipoproteins (Alkhalifah et al., 2021a). The reviewers, however stated that due to the risk of bias and limited studies in their study, the results of the review remain inconclusive. The evidence on the lipid lowering effect of honey remains inconclusive.

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Few studies have assessed the effect of white sugar and brown sugar on lipid profile. In a study by (Gómez-Crisóstomo, de la Cruz Arias, Camacho Liévano, de la Cruz-Hernández, & Martínez-Abundis, 2017) 30% solution of white and brown sugar were administered for 6 months in male rats. The consumption of white sugar resulted in high levels of triglycerides and fat build-up in the liver as opposed to brown sugar. In contrast, different doses (2500 and 5000 mg/kg) of cane brown sugar did not have any significant effect on blood lipids (TC and TG) in Wister rats after 4 weeks of treatment (Karaye et al., 2018). One gram (1000 mg) of carbohydrate from sucrose, fructose and other natural sweeteners induced hepatic lipid accumulation after 8 weeks of daily gavage in Wistar rats (Valle et al., 2020b). Based on the conducted studies, refined sugar has a negative effect on dyslipidaemia.

Low/No caloric sweeteners have been suggested to influence lipid profile although its exact mechanism remains unclear. A meta-analysis on the effect of stevia-based sweeteners and other non-caloric sweeteners on lipid profile was conducted. Intake of stevia-based and artificial sweeteners was not associated with the lipid profile in adults although a sub-group analysis revealed that intake of the non-nutritive sweeteners was associated with little but a significant increase in LDL (Movahedian et al., 2021a). Similarly, the positive health effect of replacing sugar sweetened beverages with LNCS was reiterated in a meta-analysis by (McGlynn et al., 2022) in which substitution of sugar sweetened beverages with low or no calorie sweeteners showed reduced intrahepatocellular fat deposits. In evaluating the effect of substituting sucrose for stevia, stevia at a dose of 500 mg/kg bw per day for 12 weeks caused a decrease in TC, TG, LDL-C and a rise in HDL-C (Abo Elnaga et al., 2016). Eight (8) weeks-old mice were administered with water sweetened with sucrose and sucralose for 16 weeks after which their metabolic effects were assessed. Mice given sucralose-sweetened water had a less profound influence on plasma lipids than mice given sucrose-sweetened water (Wu et al., 2023). Similarly, 18 healthy subjects in a randomized controlled trial, received intragastric

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administration of LNCS (25g D-allulose and 50g erythritol). After 3 study visits by the participants it was observed that both sweeteners had no impact on blood lipids (Teysseire et al., 2023). After 4 weeks of treatment, total cholesterol and triglycerides were lower in streptozotocin-induced diabetic rats fed a high-fat diet and administered with aqueous stevia leaf extract (Kamal et al., 2022). Stevioside supplementation for 24 weeks in diabetes patients was reported to have improved individual lipid parameters (Rashad et al., 2019).

Most studies on the effect of sweeteners on dyslipidaemia focused on individual lipid parameters. Erejuwa et al. (2016b) on the other hand emphasized on the use of lipid ratios as opposed to individual lipid parameters as a better indicator in predicting atherogenicity and metabolic syndrome. There is variation in the dosages used in the different studies.

2.6.3 Overweight and Obesity

Obesity is a risk factor for several chronic conditions, including hypertension, CVD, diabetes, dyslipidaemia, and several malignancies. Globally, its prevalence has more than doubled, coinciding with an increase in noncommunicable disorders such as metabolic syndrome (Veit et al., 2022a). It was estimated in 2022 that, 2.5 billion adults (18 years and older) were either overweight or obese and over 390 million children had either overweight or obesity (World Health Organisation, 2024). Sixty percent of Europeans are either obese or overweight, with the highest prevalence in women, and increasing with age in both sexes (Boutari & Mantzoros, 2022). Based on data from the Organization for Economic Cooperation and Development (OECD) the highest prevalence is in the US, Mexico, New Zealand and Hungary and the lowest in Japan, Korea, Italy and Switzerland (OECD, 2017).

It is the main cause of mortality, premature disability and non-communicable diseases such as CVD (hypertension, myocardial infarction and stroke), metabolic conditions (type 2 diabetes, fatty liver) and cancer (Blüher, 2019). Although genetics may play a part in its pathogenesis,

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dietary intake is its main risk factor; with energy intake exceeding energy output through reduced physical activity and energy expenditure (Kayode & Kayode, 2021; Parmar & Can, 2023; Blüher, 2019). Westernization, industrialization and urbanization have resulted in a nutrition transition towards the intake of diets high in fat and sugars with little nutritional value (Kayode & Kayode, 2021). In the US, data on the intake of sugars is sufficient in explaining the changes in adult obesity trend over the past 30 years with a cumulative effect of increased calories from sugar over time (Atangwho et al., 2020a). Not only does excessive intake of sugars increase caloric intake, but also it displaces the other essential nutrients in the diet (Atangwho et al., 2020a). Due to the evidence linking the intake of caloric or nutritive sweeteners to the obesity epidemic, there is the introduction of LNCS as a replacement to contribute to the sweet taste without adding on to the caloric content of meals. Recent research in both humans and animals suggests that LNCS may result in an increased risk of obesity and metabolic disorders, while the exact mechanism is unknown (Walbolt & Koh, 2020a).

One of the five clinical variables used to diagnose metabolic syndrome is obesity (Paley & Johnson, 2018) and it is due to excessive accumulation of fat. Obesity is defined as having a BMI larger than or equal to 30 kg/m^2, whereas overweight is defined as having a BMI greater than or equal to 25 kg/m^2 (World Health Organization, 2021). Despite the usefulness of BMI as a surrogate marker in predicting cardiovascular and metabolic disease risk, BMI only measures excess weight without giving an indication of fat distribution in the body and was also not able to fully capture cardiometabolic risk since it is not able to measure abdominal obesity (Ross et al., 2020). As simple as it is, waist circumference is a useful indicator in assessing abdominal obesity and has been reported as good predictor for cardiovascular and metabolic syndrome even after adjusting for BMI (Ross et al., 2020). The International Atherosclerosis Society (IAS) and the International Chair on Cardiometabolic Risk (ICCR) in 2017 issued a consensus statement recommending that BMI should not be used as a stand-alone

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indicator in assessing or diagnosing cardiometabolic risk but should be used together with waist circumference in assessing weight gain. The current recommendations suggest a cut-off of more than 102 cm for men and more than 88 cm for women (Ross et al., 2020). Ethnic specific cut-off for BMI and waist circumference is being proposed due to racial differences. Excess abdominal fat (visceral or intraperitoneal) has been linked to insulin resistance, decreased glucose tolerance, dyslipidaemia, elevated blood pressure, and mortality from CVD. High visceral fat has been linked to an increase in the release of fatty acids into the portal system, which increases hepatic production of triglycerides, small and dense low-density lipoprotein, and plasma insulin levels (Castro et al., 2003).

2.6.3.1 Intake of Sweeteners and Obesity

Sucrose, as the main type of carbohydrate in caloric sweeteners, is cleaved into glucose and fructose units. Glucose and fructose, which are end products in the digestion of sweeteners have both direct and indirect effect on the pathogenesis of obesity. Glucose stimulates insulin release which facilitates the conversion of excess glucose storage in the liver and muscles as glycogen and stored as fat in the liver, muscles, and adipose tissue. Fructose has been linked to obesity indirectly by causing overeating by suppressing post prandial ghrelin secretion and causing leptin resistance leading to increased caloric intake and contributing to weight gain (Johnson, Sánchez-Lozada, Andrews, & Lanaspa, 2017; Shapiro et al., 2008). Not only does it suppress satiety, but it also increases the palatability of food causing overeating and a consequent weight gain. It has also been reported to promote the storage of fat by increasing circulating triglycerides, visceral fat stores, reducing fat oxidation and the production of energy from food stores (Shapiro et al., 2008).

Excessive consumption of refined sugars, sucrose, and fructose was linked to long-term weight increase in men in a Japanese prospective cohort research (Yamakawa et al., 2020). Among

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children and adolescents, a systematic review assessing the relationship between non-nutritive sweeteners (NNS) on BMI showed that compared to sugar, NNS had a lesser effect on BMI increase in adolescent and obese individuals (Espinosa et al., 2023). Shamsi-Goushki et al. (2021b) administered 15% of brown sugar and white sugar to Wistar rats for 42 days. Both brown and white sugar contributed to significant weight change in the rats, however the effect was more pronounced in white sugar than brown sugar. Atangwho et al. (2020a) in an animal model fed male and female Wistar rats with a diet incorporated with different doses of both honey and table sugar (8% and 16%) and honey (10% and 20%) in rats for 29 weeks. Among female Wistar rats, high concentrations of both honey and table sugar had a significant effect on weight gain in comparison with the control which buttresses the relationship between high intake of sugars and its effect on weight gain, however the observed weight gain was higher in 20% honey than the 16% table sugar although there was no significant difference in the weight of the white adipose tissue mass in both groups. Conversely in the male Wistar rats, weight was higher in the rats fed with 8% table sugar incorporated than the 10% honey-based diet and the control group. The obesogenic effect of sweeteners was concurred in another study by Valle et al. (2020b) in which both natural and refined sugars caused weight gain in Wistar rats after 8 weeks of daily gavage. Similarly, in another animal model, white sugar and brown sugar were administered to rats by dissolving it in their drinking water for six months. Both refined sugar and brown sugar induced weight gain with the retroperitoneal fat almost doubling with no significant difference in their effects. Sánchez-Tapia et al. (2020a) in their study examined the effect of different types of sweeteners (caloric and non-caloric) on metabolic parameters. Sucrose, fructose, glucose, brown sugar, honey, sucralose, and stevia were dissolved in the drinking water of Wistar rats for 4 months. The caloric sweeteners had more microbial diversity in the presence of high fat diet compared with the non-caloric sweeteners. Sucrose administration had the most effect on weight gain. A high-fat and high-sugar diet increases

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intestinal bacteria linked to obesity (Davis, 2016). To compare the effect of both caloric and non-caloric sweeteners on metabolic parameters without missing the effect of gender, male and female rats were administered sucrose, stevia, and sucralose through their drinking water for 16 weeks. The rats preferred sucralose and stevia sweetened water to sucrose. Weight gain in all sweetener groups did not increase compared with the control group (Farid et al., 2020). Barrios-Correa et al. (2018a) administered commercial sweeteners (sucrose, stevia, sucralose) to male and female mice for 6 weeks through their drinking water. Water intake was higher in the sucrose sweetened group than in other sweetener groups in both male and female mice, suggesting a preference for sucrose. Supplementation with stevia was associated with a reduction in energy intake in male mice but not female mice. There was a significant increase in body weight in male mice administered with stevia compared with the control however in the female mice, sucrose and sucralose supplementation induced weight gain, suggesting that stevia had a weight-lowering effect whereas sucralose had an effect on weight gain. Supplementation with a stevia extract in diabetic induced rats showed significant reduction in the weight of the stevia group when compared with the normal and diabetic controls (Ahmad & Ahmad, 2018b). A similar finding was reported by Abo Elnaga et al. (2016) with stevia sweetener supplementation associated with reduced feed intake and body weight. Similarly, xylitol and erythritol administered to diabetic induced Sprague Dawley rats for 8 weeks reduced body weight in the experimental animals (Msomi et al., 2023). In another animal model to assess the effect of caloric and non-caloric sweeteners on metabolic health, there was no difference in weight gain between both the intervention groups and the control, neither was there a difference in the retroperitoneal fat pad weights among the treatment groups (Figlewicz et al., 2009c).

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CHAPTER THREE

3.0 METHODOLOGY

3.1 EXPERIMENTAL DESIGN

An experimental study design was used. A control/ treatment group design was used.

3.2 STUDY SITE

The study was conducted at the Department of Animal Experimentation (DAE) at the Noguchi Memorial Institute for Medical Research, University of Ghana (NMIMR).

3.3 STUDY ANIMALS AND SAMPLING

Seventy-eight (78) female Sprague Dawley rats aged 10-12 weeks, weighing between 100 g – 200 g were obtained from the DAE at the NMIMR. The rats were weighed and randomized into thirteen (13) groups of six (6) animals in each cage. The animals were kept in rat cages with soft wood shavings as bedding and acclimatized to their environment for a week. The experimental groups were set up as follows: Group 1- Control (C), Group 2 – White sugar low dose (WSLD), Group 3- white sugar medium dose (WSMD), Group 4 – white sugar high dose (WSHD), Group 5 – Brown sugar low dose (BSLD), Group 6 – Brown sugar medium dose (BSMD), Group 7 – Brown sugar high dose (BSHD), Group 8 – Honey low dose (HLD), Group 9 – Honey medium dose (HMD), Group 10 – Honey high dose (HHD), Group 11 – stevia low dose (SLD), Group 12 – Stevia medium dose (SMD), Group 13 – Stevia high dose (SHD) (Figure 3.1). Administration of the sweeteners was done for 17 weeks.

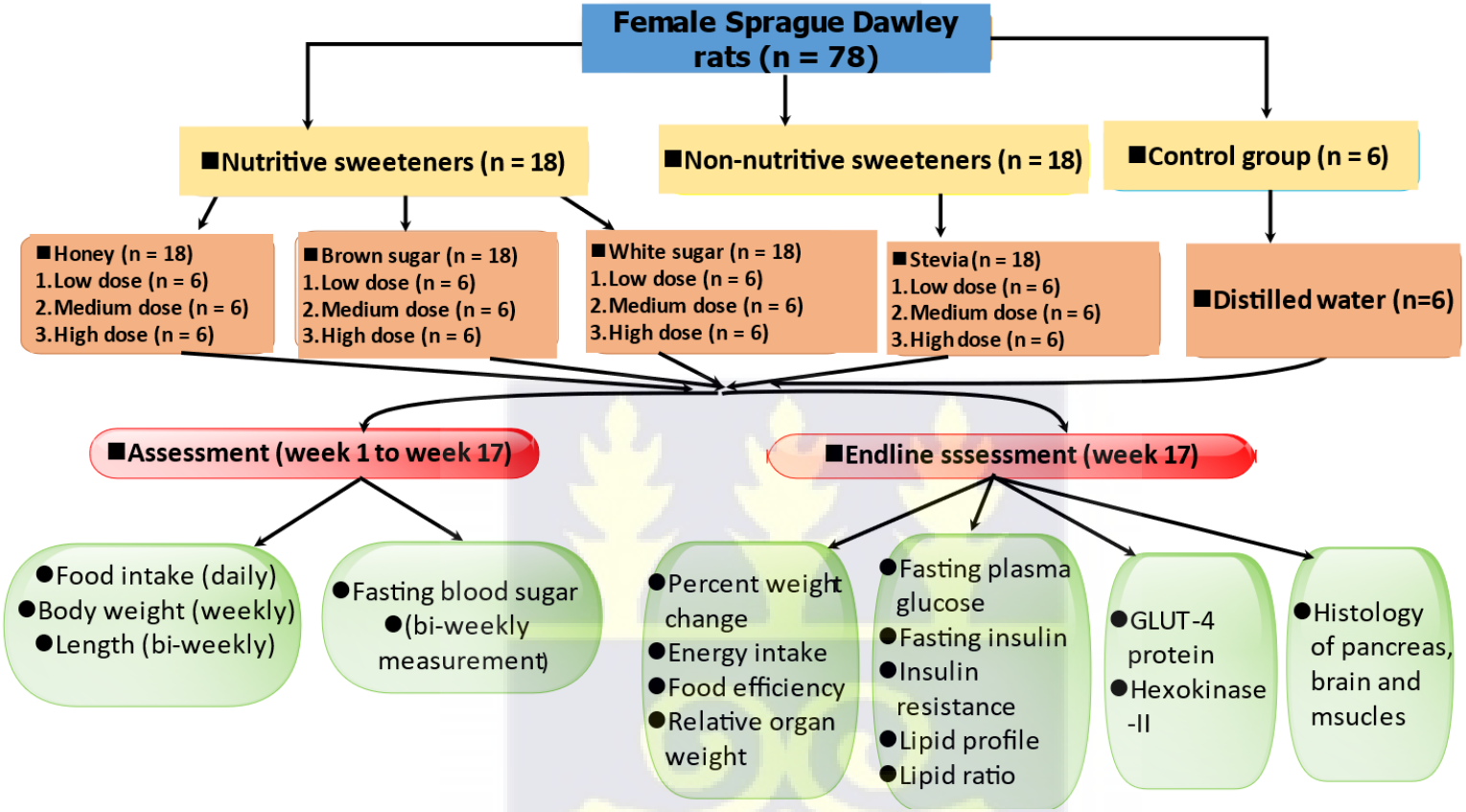

Figure 3.1: Study Design A total of 78 female SD rats were divided into 13 groups of 6 animals, including a control group. Bi-weekly fasting plasma glucose was assessed by tail clipping. After 17 weeks of administration, all 78 rats were euthanised and blood was collected by cardiac puncture.

INTEGRI PROCEDAMUS

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3.4 ETHICS

The study was approved by the Institutional Animal Care and Use Committee of University of Ghana (UG-IACUC 001/21-22) (Appendix M). All animal procedures and techniques used in this study were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (National Research Council, 2011). Additionally, animals were treated humanely.

3.5 HOUSING AND FEEDING OF EXPERIMENTAL ANIMALS

Rats were kept at the DAE throughout the study under the following standard conditions of temperature (24ºC ± 2ºC) and relative humidity (60-70%) with a 12-hour light/dark cycle. The animals were fed on standard rat chow and distilled water *ad libitum* for one week before the commencement of the experiment. Rat chow was formulated and supplied by Agricare limited (Kumasi, Ghana).

3.6 DETERMINATION OF DIET REGIMEN OF SWEETENERS

The percentage of sugar solutions for nutritive sweeteners was calculated according to the WHO recommendation of reducing the intake of free sugars to less than 10% of total energy intake and a further reduction of the intake of free sugars to below 5% of total energy intake (World Health Organisation, 2018). Thus, for an average adult (70 kg) with a calorie intake of 2000 kcal, 5% of total energy intake is equivalent to 100 kcal. For this study, the dosages were categorised into low doses (5% of total energy intake), moderate doses (10 % of total energy intake) and high doses (15 % of total energy intake). Thus, the weight of each nutritive sweetener to provide 100 kcal was estimated from their proximate analysis (Table 3.1). For example, 25 g of white sugar was equivalent to 100 kcal as follows:

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For a 70 kg adult requiring 2000 kcal, low dose = 25 g/70 kg = 0.36 g/kg body weight

Thus, for a 200 g (0.2 kg) rat, the required dosage = 0.2 kg x 0.36 g/ kg = 0.072

0.072 g of the white sugar was dissolved in 2 ml of distilled water.

A stock solution was prepared at 0.035 g/ml for low-dose white sugar.

Moderate (10 % of total energy intake) = 0.07 g/ml

High dose (15 % of total energy intake) = 0.1 g/ml

Table 3.1: Sweetener Dosages

Group No	Group	No. of Rats	Feed composition	Dosage (g/ml)
1	Normal Control (C)	6	Distilled water	
2	White Sugar Low Dose (WSL)	6	Normal Chow + 5% white sugar	0.035
3	White Sugar Medium Dose (WSM)	6	Normal Chow + 10% white sugar	0.07
4	White Sugar High Dose (WSHD)	6	Normal Chow + 15% white sugar	0.1
5	Brown Sugar Low Dose (BSLD)	6	Normal Chow + 5% brown sugar	0.036
6	Brown Sugar Medium Dose (BSMD)	6	Normal Chow + 10% brown sugar	0.072
7	Brown Sugar High Dose (BSHD)	6	Normal Chow + 15% brown sugar	0.11
8	Honey Low Dose (HLD)	6	Normal Chow + 5% honey	0.047
9	Honey Medium Dose (HMD)	6	Normal Chow + 10% honey	0.094
10	Honey High Dose (HHD)	6	Normal Chow + 15% honey	0.14
11	Stevia Low Dose (SLD)	6	Normal Chow + 5% stevia	0.007
12	Stevia Medium Dose (SMD)	6	Normal Chow + 10% stevia	0.014
13	Stevia High Dose (SHD)	6	Normal Chow + 15% stevia	0.021

3.7 FEED, ENERGY AND WATER INTAKE

Food intake was measured daily throughout the study. The mean quantity of chow intake per experimental group was determined by measuring their daily food intake, based on the administration of a known quantity of chow per cage (200 g) and the weight of the leftover chow after 24 hours. Spilt rat chow in each cage was carefully collected and added to the weight of

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leftover chow in the feeding troughs. Total energy consumed was calculated at the end of the study by multiplying the amount of food consumed by the caloric contents per gram of chow as stated by the manufacturer (2.89 kcal/g) (Barrios-Correa et al., 2018b). Food efficiency was calculated using the equation by (López-Varela, Sánchez-Muniz, & Cuesta, 1995):

$$\text{Food efficiency} = \frac{\text{body weight gain (g)}}{\text{food intake (g)}} \times 100$$

Water was provided *ad libitum* daily.

3.8 WEIGHT OF RATS

The rats were weighed weekly at the same time using an animal scale (Shinano, TS-870). The weighing bowl was sanitized with 70% ethanol and placed on the scale. The rat was gently placed in the weighing bowl. The reading was allowed to stabilize before the animal's actual weight was recorded. Percent weight gain was calculated as indicated below:

$$\text{Percent weight change} = \frac{\text{end line weight-initial weight}}{\text{initial weight}} \text{ x } 100$$

3.9 EUTHANASIA AND BLOOD DRAW

At the end of the 17 weeks the rats were fasted overnight, and a blood draw was done by cardiac puncture based on the institutional protocol at DAE. This is a terminal procedure used to permit the collection of larger amounts of blood. The experimental animals were anaesthetized using diethyl ether. The rats were placed on their back. The left index finger was placed at the level of the lowest ribs without applying any pressure. The heart was gently located approximately 1 cm above this point, slightly to the right. Using a 5 ml syringe with a 23 G needle, the needle was inserted between the two ribs at an angle of 45°C. An amount of 2 ml of blood was put into lithium heparin tubes and 1 ml of blood was discharged into fluoride tubes and EDTA tubes. These

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techniques were done in the presence of a veterinarian and a veterinary technician who assessed that the animals were handled properly and not subjected to unnecessary pain or distress (Parasuraman et al., 2010). Blood samples in lithium heparin and fluoride tubes were centrifuged at 3000 rpm for 10 minutes to separate the plasma. Plasma samples were transferred into Eppendorf tubes, labelled, and stored in a laboratory freezer at -80°C until analysis.

3.10 ORGAN HARVESTING AND RELATIVE ORGAN WEIGHT

At the end of 17 weeks, the rats were euthanised and the pancreas, kidney, heart, and liver were harvested. The relative organ weight (ROW) of each organ was then calculated as reported by (Samat, Nor, Nor Hussein, & Ismail, 2014):

$$\text{ROW} = \frac{\text{absolute organ weight (g)}}{\text{body weight on sacrifice day (g)}} \times 100$$

The harvested organs were divided into two parts; one part was preserved in 10% buffered formalin and the other half immediately snap frozen with liquid nitrogen and subsequently stored at -80°C for subsequent laboratory analysis. The soleus and gastrocnemius muscle were harvested immediately snap frozen and subsequently transferred to -80°C to be used for the Hexokinase 2 analysis.

3.11 BIOCHEMICAL ANALYSIS

3.11.1 Measurement of Fasting Plasma Glucose

The fasting plasma glucose of the rats was measured bi-weekly by tail clipping using Accu-Chek Active Blood Glucose Glucometer (Mannheim, Germany). The distal third of the tail was wiped with a ball of cotton wool moistened with 70% alcohol. In the non-dominant hand, the tail was gently stroked down from the base of the tail to about 1 cm from the tip of the tail. The tip of the

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tail was placed on a working surface and with the dominant hand, a needle prick was performed using the 25 G needle about 1-2 mm from the end. The blood sample was collected from the drop of blood that formed at the skin surface onto the glucometer test strip and the reading was done. The pressure in the non-dominant hand was released and gentle pressure was applied with a small ball of cotton wool for approximately 10 seconds to stop the flow of blood. The tail tip was monitored for re-bleeding and the rat was returned to its cage (McErlane, 2014). Three (3) rats from each group were used for the bi-weekly fasting plasma glucose test in an alternating manner.

3.11.2 Lipid Profile

Plasma lipid profile consisting of total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) were analysed with an auto-analyser machine and test kits according to the manufacturer's instructions (Mindray Bio-Medical Electronics Bs-200e, Shenzhen, China).

3.11.2.1 Lipid ratios

Lipid ratios were calculated from the values obtained from the lipid profile; Castelli Risk Index I = TC/ HDL-C; Castelli Risk Index II = LDL/ HDL-C; Atherogenic Index of Plasma = Log (TG/HDL-C) (Uzunget & Sahin, 2022).

3.11.3 Fasting Insulin Level

Rat heparinised plasma was used in measuring the insulin level. Fasting insulin level was determined using a 96-plate rat insulin Elisa kit purchased from Elabscience (Product No: E-EL-R3034, Lot No: kl1OXBOB6381) (Houston, Texas, USA) according to the manufacturer's instruction. In summary, 100 µl of the standard and the sample were added to the wells and incubated for 90 minutes at 37°C. The liquid was discarded and 100 µl of the Biotinylated

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Detection Ab working solution was added to each well and incubated for 60 mins at 37°C. The wells were aspirated and washed 3 times. After which 100 µl horseradish peroxidase (HRP) conjugate working solution was added and incubated for 30 minutes at 37°C. The plate was aspirated and washed 5 times. A volume of 90 µl of the substrate reagent was added to the wells and incubated for 15 minutes at 37°C. Finally, a 50 µl Stop solution was added to the wells to stop the enzyme-substrate reaction which turned the colour to yellow. The Optical Density (OD) of the plate was measured immediately at a wavelength of 450 nm using a BioTek plate reader Microplate Photometer (BioTek Instruments, Inc, USA). The OD was proportional to the concentration of the rat fasting insulin. The rat insulin concentration was calculated by comparing the OD of the samples to the standard curve.

3.11.3.1 Insulin resistance

The homeostatic model for insulin resistance was used to determine the insulin resistance; (fasting plasma insulin µU/ml x fasting plasma glucose (mmol/l) / 22.5 (Gayoso-Diz et al., 2013c).

3.12 GLUT4 ANALYSIS

GLUT4 protein was measured using a rat GLUT4 Elisa Kit purchased from MYBioSource (San Diego, USA) according to the manufacturer's instruction. This kit uses the sandwich enzyme-linked immune-sorbent assay (ELISA) technology. In summary an antibody specific for GLUT4 had been pre-coated onto the 96-well microplate. Hundred microliters of the standard and samples were added to the wells and incubated at 37°C for 2 hours. Any GLUT4 protein present in the samples were bound by the antibody and the liquid in the wells discarded. Hundred microliters of a biotin-conjugated antibody specific to GLUT4 was added to the wells and incubated at 37°C for 1 hour. Afterwards the liquid in the wells were aspirated and washed. Horseradish Peroxidase (HRP) (100 µl) was added to the wells and incubated at 37 °C for an hour. TMB (90 µl) Substrate

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were added to the wells which was catalysed by HRP to produce a blue colour after incubating at 37°C for 15-30 minutes. A stop solution (50 µl) was added to each well which changed the colour of the wells to yellow. The Optical Density (OD) of the plate was measured immediately at a wavelength of 450 nm using a BioTek plate reader Microplate Photometer (BioTek Instruments, Inc, USA). The OD was proportional to the concentration of GLUT4 protein. The GLUT4 concentration was calculated by comparing the OD of the samples to the standard curve.

3.13 HEXOKINASE 2 ANALYSIS

Hexokinase II in the gastrocnemius muscle was measured using a rat hexokinase 2 Elisa kit from Abbexa (Cambridge, United Kingdom). The muscle tissue was allowed to thaw and homogenized manually. The muscle tissue (0.5 g) was minced on a plastic petri dish for 3 minutes. The minced tissue was homogenized in a test tube using a manual glass tissue grinder (250 strokes). A phosphate buffer was used as the diluent at a pH of 7.4. All tissues were minced and homogenized on ice to prevent loss of the hexokinase enzyme. The homogenized muscle tissues were transferred into Eppendorf tubes and centrifuged at 13000 rpm for 4 minutes at 4^{o}C. The supernatant was collected and stored at -20^{o}C for the assay.

The supernatant, standard diluent buffer and diluted standards (100 µl) were added to the pre-coated plate and their positions labelled. The plate was incubated at 37 °C for an hour. The liquid in the wells were discarded afterward and 100 µl of the detection Reagent A working solution was added to each well. It was incubated at 37°C for one hour. After incubation, the plate was washed 3 times by filling each well with 350 µl of wash buffer. Detection Reagent B working solution was added to each well and incubated at 37°C for 30 minutes. The solution was discarded afterward, and the plate washed 3 times using the wash buffer. The TMB Substrate (90 µl) was aliquoted into each well and incubated at 37°C for 20 minutes. The presence of Hexokinase 2 generated a blue

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colour. The stop solution (50 µl) was added to each well and mixed quickly and uniformly to ensure the enzyme is completely deactivated. Addition of the stop solution produces a yellow colour. The intensity of the yellow colour was proportional to the amount of hexokinase II in the wells. The Optical Density (OD) was measured at 450 nm using a BioTek plate reader Microplate Photometer (BioTek Instruments, Inc, USA). Hexokinase II concentration was calculated by comparing the OD of the samples to the standard curve. The relative OD was calculated as follows:

Relative OD=OD of each well-OD of zero well

The standard curve was then plotted as the relative OD of each reference standard solution (X), against the respective concentration of each standard solution (Y). The concentration of the samples was interpolated from the standard curve.

3.14 HISTOLOGICAL ANALYSIS

Samples of the liver, kidney and heart were fixed in a neutral buffered formalin to prevent degradation of the tissue. Following fixation, samples were trimmed using a scalpel to enable them properly to fit in a labelled cassette. The specimen was then dehydrated by immersing it in increasing levels of alcohol to get rid of any water or formalin present in the tissue. The alcohol was then displaced by using xylene which allow paraffin infiltration. The specimen was embedded in paraffin wax, which surrounded the tissue and created a "block" needed for further sectioning. The paraffin embedded wax was sectioned using a microtome into thin ribbons about 5 micrometres. The thin sections were transferred into a warm water bath and afterward lifted onto the slide to allow it to dry. Following the sectioning, slides were stained with haematoxylin and eosin (H&E). After the staining was completed, the slides were covered with cover slips and viewed under an Olympus microscope (United Kingdom) at times 40 magnification (x 40).

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3.15 STATISTICAL ANALYSIS

GraphPad prism software version 9.0 was used for the statistical analysis. Descriptive variables were expressed as Means ± SEM. Data that were not normally distributed were transformed using the logarithmic transformation method. Statistical significance was set at $p< 0.05$. Two-way mixed ANOVA was used to compare the changes in body weight and fasting plasma glucose over time among the various experimental groups. One-way ANOVA was used to compare differences in the means of different variables (percent weight change, end line fasting plasma glucose, fasting serum, HOMA-IR, total cholesterol, triglyceride, Low density lipoprotein, high density lipoprotein, Castelli Risk Index I and II and Atherogenic Index of Plasma) between the experimental groups. Tukey's post hoc tests were used to detect where differences between the group were observed.

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CHAPTER FOUR

4.0 RESULTS

4.1 UNEXPECTED MORBIDITY AND MORTALITY

There were no treatment-related deaths throughout the 17 weeks of the study. One rat in the honey low-dose treatment group developed an abnormality in balance. An ear swap was done to rule out infection. The result came out negative for infection. One rat in the high-dose white sugar group had an injury in the eye and another rat in the same group had an arm injury. Both rats were treated and left in the group. No apparent differences in physical activity or behaviours, no significant changes in stool, urine, eye colour, diarrhoea, salivation, convulsion, sleep or comma and no significant loss of fur or skin lesions were observed.

4.2 ANTHROPOMETRY AND FOOD INTAKE

Section 4.2 provides results on the anthropometric assessment (weekly weight, percent weight gain, relative organ weight) and food intake (energy intake and energy efficiency) of the rats.

4.2.1 Effect of Sweeteners on Body Weight of the Experimental Animals

Figure 4.1 shows the trend in weight gain across the experimental groups at baseline and end line. The average weight of the rats increased over time in all the experimental groups. At baseline, the control group had the highest average body weight (190.3 ± 7.1 g) whereas rats in the high-dose stevia group had the least average body weight (142.0 ± 3.0 g). At the end of the study, the experimental group administered with high-dose white sugar had the highest average body weight (285.3 ± 10.2 g) with the least average weight observed in the group that were administered with low-dose stevia administration (238.0 ± 8.6 g).

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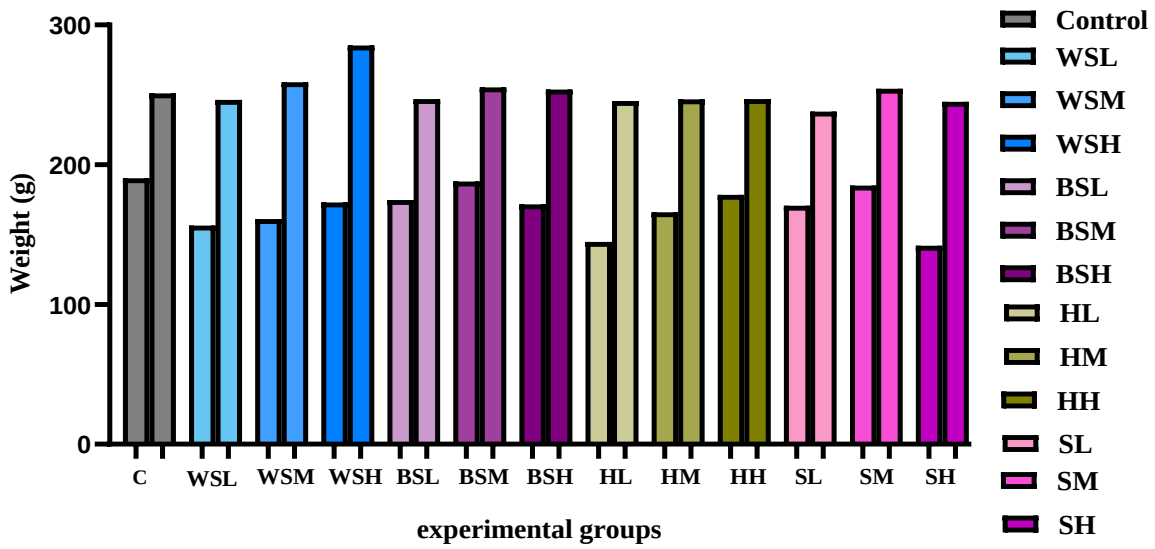

Figure 4.1: Body weight at baseline (left bar) and end line (right bar) for each experimental group. C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high.

A two-way mixed ANOVA, run to determine the effect of the type of sweetener and dosage over time on the mean weight of the experimental animals, showed a statistically significant difference in weight gain at different time points [F (2.35, 152.86) = 567.45, p<0.0001, η^2 = 0.897]. There was a statistically significant modest association between the type and dosage of sweetener and the weight gain [F (12, 65) = 2.39, p<0.013, η^2 = 0.306]. The combined effect of the duration of treatment and treatment type on weight gain was statistically significant [F (28.22, 52.86) = 2.85, p<0.0001, η^2 = 0.897)]. The duration of treatment accounted for 56.3% of the total variation in weight gain regardless of the treatment being offered after adjusting and matching whereas the treatment type accounted for 10.4% of the variation in weight gain regardless of the time.

4.2.2 Effect of Sweeteners on Percent Weight Gain

The trend in percent weight gain after 17 weeks of treatment among the experimental groups is presented in Figure 4.2. The least percent weight gain was observed in the control group (32.2 ±

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11.3%) and highest in the experimental group administered with high dose stevia (72.7 ± 10.5%). A dose-dependent increase in the percent weight change was observed in white sugar whereas a dose-dependent decrease was observed with honey administration.

A one-way ANOVA showed a significant association between the type and dosage of sweetener administered and the percent weight gain in the rats [($F(12, 65) = 3.953$, $p<0.001$, $\eta^2=0.42$)], indicating that the sweeteners had an impact on weight gain. Forty-two percent (42%) of the variance in percent weight gain was accounted for by the type and dosage of sweetener administered. A post hoc test revealed statistically significant differences in per cent weight gain between several groups.

Among the experimental groups administered with low dosages, honey had a significantly higher percent weight gain than the control group ($p=0.002$). In comparing the experimental groups administered with medium doses of the various sweeteners, significant differences were observed in their percent weight gain [$F (2, 12) = 7.31$, $p=0.007$]. The experimental group administered with medium-dose white sugar had a significantly higher percent weight gain than the groups administered with medium-dose brown sugar ($p=0.029$) and medium-dose stevia ($p=0.039$). In comparing the experimental groups administered with high doses, there was a statistically significant differences in their percent weight gain [$F (2, 10) = 6.44$, $p=0.016$)]. The experimental group administered with a high dose of stevia had a significantly higher percent weight gain than the control group ($p=0.029$) and the group administered with high dose of honey ($p=0.039$). Also,

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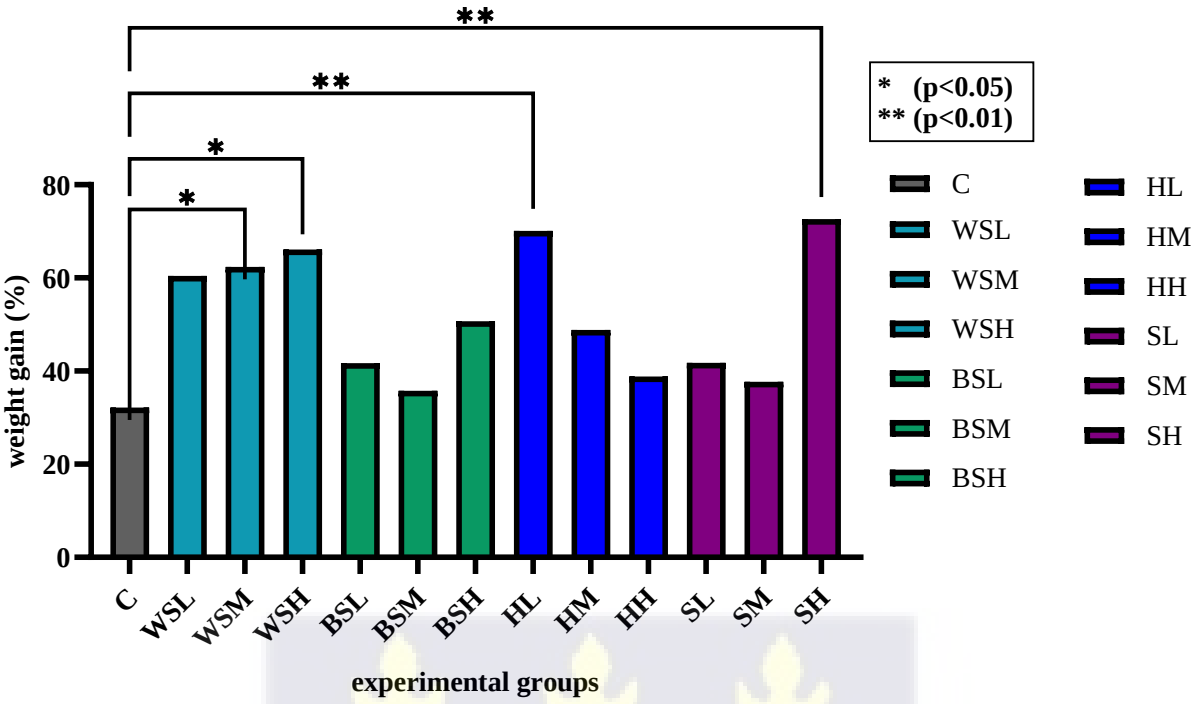

Figure 4.2: Effect of sweeteners on percentage weight change after 17 weeks of treatment. C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high.

the group administered with high-dose white sugar also had a significantly higher weight gain than the control group (p=0.014).

Within the individual sweetener groups, there was no significant differences in the percent weight gain with different doses of brown sugar and white sugar. However, among the honey administered groups, rats administered with low-dose honey had a significantly higher percent weight gain compared with the control group (p<0.001), medium-dose honey (p=0.015), and high-dose honey (p<0.001). Among the experimental groups administered with stevia, rats administered with high-dose stevia had a significantly greater percent weight gain than the control group (p=0.005), medium-dose stevia (p=0.034) and low-dose stevia (p=0.015).

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4.2.3 Relative Organ Weight of Rats After 17 Weeks of Treatment

Table 4.1 shows the relative organ weight of the experimental animals. The experimental group administered with low-dose honey had the highest relative heart weight (0.40 ± 0.07 g) whereas the control group had the least relative heart weight (0.28 ± 0.06 g). The relative liver weight was similar in all the experimental groups. Most of the study group animals had similar relative kidney weights. A similar observation was made with the relative brain weight. Among the experimental groups administered with white sugar, brown sugar and stevia, there was an observed dose-dependent decrease in the relative brain weight. However, there were no significant differences in the relative heart, kidney, liver, and brain weights within the different treatment group.

4.2.4 Total Energy Intake and Food Efficiency

The total energy intake among the experimental animals over 17 weeks is shown in Figure 4.3. The total energy intake was highest in rats administered with high-dose white sugar (461.9 kcal) and least in rats administered with medium-dose honey (362.2 kcal). There was a dose-dependent increase in the total energy intake of groups that were administered with white sugar, brown sugar, and stevia. However, this effect was not observed in the groups administered with honey.

A similar trend was observed in the Food Efficiency Ratio (FER) (Figure 4.4). The FER was highest in the rats that were administered with high dose white sugar (69.9 %) and lowest in the control group (43.4%). There was a dose-dependent increase in the FER with groups of white sugar, and stevia. Among the honey-administered groups, there was a dose-dependent decrease in FER. There was no observed trend with FER among brown – sugar administered groups.

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Table 4.1: Relative organ weight (g) of rats after 17 weeks of treatment

Groups	Heart	Liver	Kidney	Brain
C	0.28 ± 0.06	0.03 ± 0.00	0.33 ± 0.01	0.71 ± 0.03
WSL	0.34 ± 0.02	0.03 ± 0.00	0.30 ± 0.01	0.70 ± 0.03
WSM	0.35 ± 0.01	0.03 ± 0.00	0.36 ± 0.02	0.68 ± 0.02
WSH	0.34 ± 0.02	0.03 ± 0.00	0.35 ± 0.02	0.60 ± 0.05
BSL	0.36 ± 0.01	0.03 ± 0.00	0.35 ± 0.01	0.69 ± 0.03
BSM	0.37 ± 0.01	0.03 ± 0.00	0.36 ± 0.02	0.63 ± 0.07
BSH	0.34 ± 0.02	0.03 ± 0.00	0.32 ± 0.01	0.60 ± 0.03
HL	0.40 ± 0.07	0.03 ± 0.00	0.31 ± 0.01	0.68 ± 0.03
HM	0.32 ± 0.01	0.03 ± 0.00	0.33 ± 0.01	0.67 ± 0.03
HH	0.37 ± 0.02	0.03 ± 0.00	0.31 ± 0.01	0.75 ± 0.03
SL	0.37 ± 0.02	0.03 ± 0.00	0.33 ± 0.02	0.75 ± 0.04
SM	0.32 ± 0.01	0.03 ± 0.00	0.32 ± 0.01	0.66 ± 0.02
SH	0.36 ± 0.01	0.03± 0.00	0.33 ± 0.01	0.66 ± 0.04
p*-value	0.423	0.055	0.117	0.113

Results are presented as means ± SEM, n=6, One-way ANOVA, C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high

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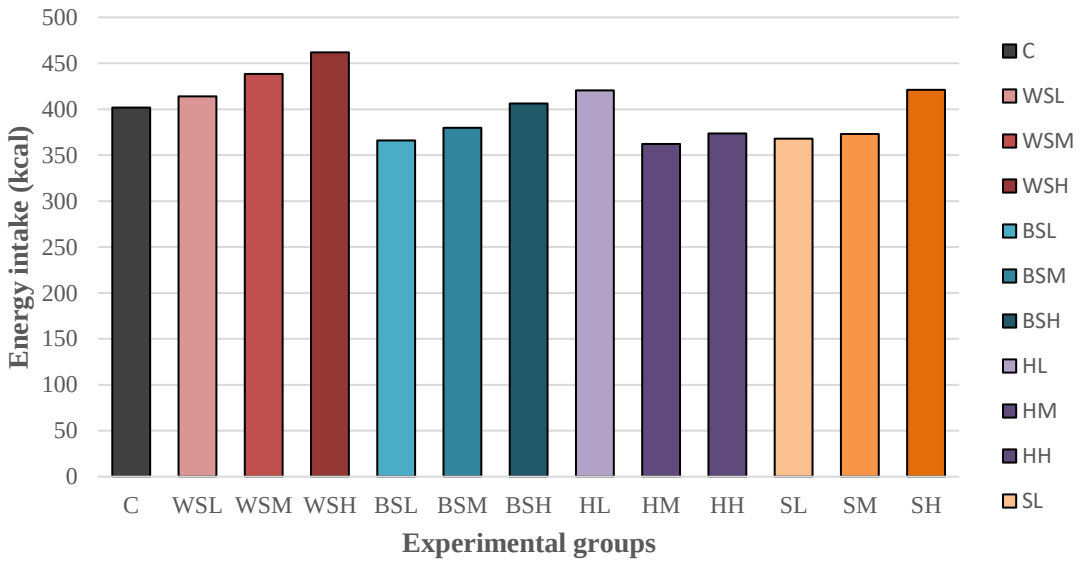

Figure 4.3: Energy intake after 17 weeks of treatment. C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high

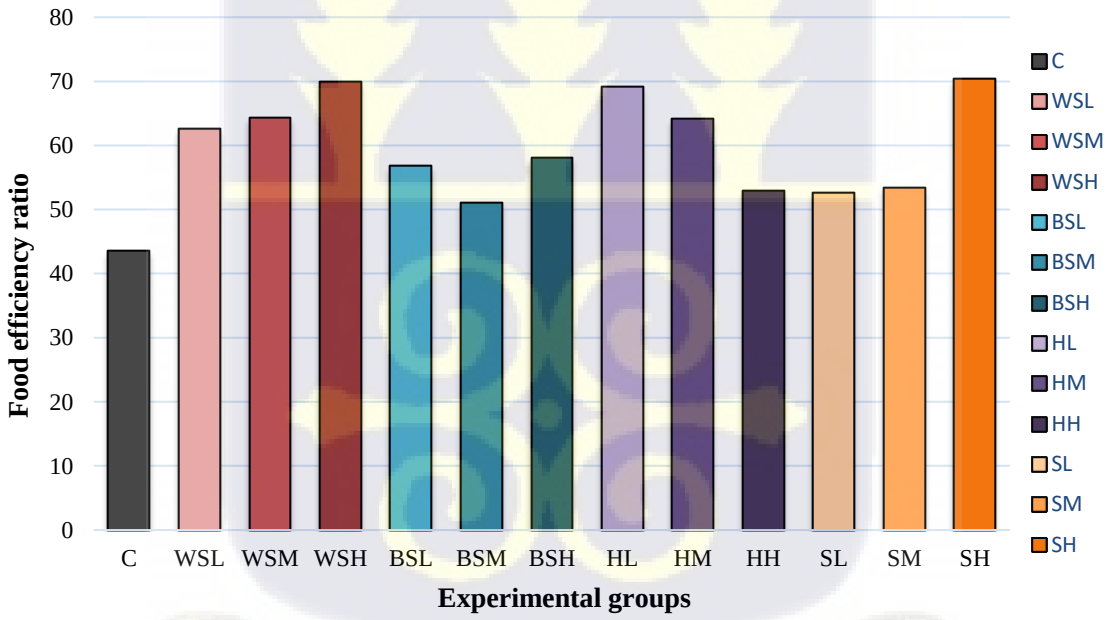

Figure 4.4: Food Efficiency Ratio After 17 Weeks of Treatment. C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high.

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4.3 EFFECT OF SWEETENER INTAKE ON BLOOD GLUCOSE PARAMETERS

4.3.1 Effect of Sweeteners on Fasting Plasma Glucose

A two-way repeated measure ANOVA revealed that main effect of time on FPG was found to be significant [$F(5, 200) = 56.95$, $p<0.001$, $\eta^2=0.594$]. There were significant differences in the fasting plasma glucose across the different time points. Fasting Plasma Glucose changed over time, regardless of the treatment group. The main effect of the treatment type (type of sweetener and dosage) on FPG was also highly significant [$F(12, 39) = 7.62$, $p<0.001$, $\eta^2=0.701$]. Fasting plasma glucose differed significantly between the different treatment groups irrespective of the duration of treatment.

The combined effect of time and sweetener type and dosage on FPG was statistically significant [$F(5,200)=56.95$, $p<0.001$, $\eta^2=0.467$]. The effect of time on the fasting plasma glucose differs depending on the type and dosage of sweetener being administered and vice-versa. The duration of treatment accounted for 37% of the total variation in the FBG of the rats irrespective of the treatment regimen and the type and dosage of sweetener accounted for 11% of the total variance observed in the FBG of the rats irrespective of the duration of treatment.

At end line, the group administered with medium-dose white sugar had the highest FPG (5.5 ± 0.2 mmol/l) whereas high-dose honey had the least FPG (4.4 ± 0.2 mmol/l) (Figure 4.5). Among the experimental groups administered with low dosages of sweeteners, rats administered with low-dose brown sugar had a significantly higher FPG than rats administered with low-dose of white sugar (p=0.002) and honey (p=0.003) but not stevia. Among the experimental groups administered with medium doses of sweeteners, rats administered with medium dose white sugar had a significantly higher FPG than rats administered with medium dose honey (p = 0.013) and medium dose stevia (p<0.001) but not rats administered with medium dose brown sugar. Comparing the

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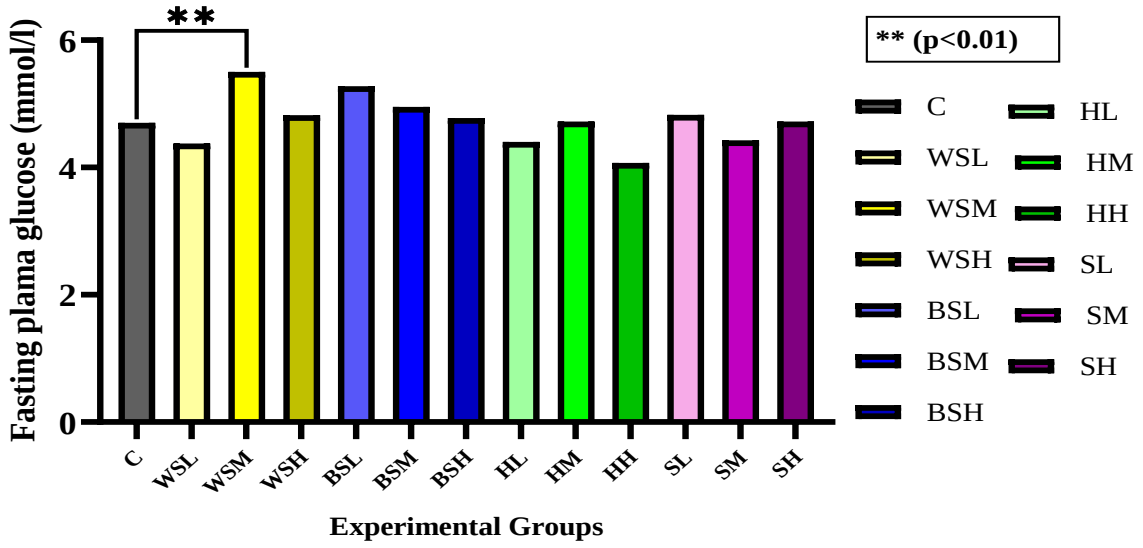

Figure 4.5: Effect of sweeteners on fasting blood glucose after 17 weeks of treatment. C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high. P value < 0.05 was considered significantly different.

experimental groups administered with high doses, rats administered with high dose of brown sugar and high dose of white sugar had the highest FPG at the end of the study which was significantly higher than rats administered with high-dose of honey (p=0.037, p=0.019, respectively) but not rats administered with high dose of stevia.

4.3.2 Effect of Sweeteners on Fasting Insulin Level

The mean fasting insulin level (13.5 ± 2.4 pg/ml) was significantly highest in the control group compared to the rest of the experimental groups (p<0.0001). Low-dose honey had the lowest insulin concentration (0.9 ± 0.1 pg/ml) (Table 4.2). A one-way ANOVA showed a significant

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Table 4.2: A One-way Anova and Post Hoc on the Effect of Sweeteners on Fasting Insulin Level

Insulin (pg/ml)	Low Dose **Mean ± SEM**	Medium Dose **Mean ± SEM**	High Dose **Mean ± SEM**	***p-value***
Control		13.5 ± 2.4		
White Sugar	2.4 ± 1.1[a]	3.8 ± 1.3[a]	3.7 ± 1.0[a]	0.684
Brown Sugar	4.9 ± 0.3[a]	1.2 ± 0.3[a]	1.1 ± 0.2[a]	**<0.001***
Honey	0.9 ± 0.1[a]	2.1 ± 0.7[a]	1.3 ± 0.3[a]	0.091
Stevia	2.1 ± 1.3[a]	1.3 ± 0.3[a]	1.2 ± 0.3[a]	0.622
p-value	**0.016***	0.137	**0.008***	

Results are presented as means ± SEM, n=6, *values are statistically significant, [a] significantly different from the control, P value < 0.05 was considered significantly different.

difference between fasting insulin concentration and the different treatment groups [F (12, 53) = 11.44, $p< 0.0001$].

There was no significant difference in the fasting insulin concentrations within the groups that were administered with white sugar, honey, and stevia. However, there was a significant difference in the fasting insulin concentrations among the various groups administered with different doses of brown sugar. Specifically, the group administered with low-dose brown sugar had a significantly higher insulin concentration than the groups administered with medium-dose brown sugar ($p<0.0001$) and high-dose brown sugar ($p<0.0001$).

In comparing the experimental groups administered with low doses of the sweeteners, there was a significant difference in insulin concentration between the low-dose groups. Specifically, the rats administered with low-dose brown sugar had a significantly higher insulin concentration than rats

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administered with low-dose honey (p = 0.009). Similarly, in comparing the experimental groups administered with high dosages of sweeteners, a significant difference was observed in their insulin concentration. Specifically, rats administered with high-dose white sugar had a significantly higher insulin concentration than rats administered with high-dose of brown sugar (p = 0.013), high dose of honey (p = 0.021) and high dose of stevia (p = 0.015).

4.3.3 Effect of Sweeteners on HOMA-IR after 17 Weeks of Treatment

The control group had the highest HOMA-IR (0.33±0.11) (Table 4.3). There was a significant difference in the HOMA-IR between the various experimental groups [F (12, 53) = 4.92, p<0.0001] with a post hoc analysis detecting differences in several groups.

The group administered with low-dose white sugar had a significantly lower HOMA-IR than the groups administered with low-dose brown sugar (p=0.004), low dose honey (p=0.002) and low dose stevia (p=0.028). Among the high dosages, white sugar had a significantly higher HOMA-IR than the groups administered with high-dose brown sugar (p=0.002), high dose honey (p=0.023), and high-dose stevia (p=0.018). Generally, HOMA-IR decreased in all the experimental groups compared with the control group.

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Table 4.3: A One-way Anova and Post Hoc on the Effect of Sweeteners on HOMA-IR

	Low Dose **Mean± SEM**	Medium Dose **Mean± SEM**	High Dose **Mean± SEM**	***p-value***
Control		0.33 ± 0.11		
White Sugar	0.02 ± 0.00[a]	0.11 ± 0.04[a]	0.09 ± 0.02[a]	0.107
Brown Sugar	0.11 ± 0.02[a]	0.02 ± 0.01[a]	0.02 ± 0.00[a]	**0.001***
Honey	0.04 ± 0.02[a]	0.04 ± 0.02[a]	0.04 ± 0.01[a]	0.213
Stevia	0.04 ± 0.02[a]	0.04 ± 0.01[a]	0.03 ± 0.01[a]	0.908
p-value	**0.002***	0.097	**0.003***	

Results are presented as means (SEM), n=6, *values are statistically significant, [a] significantly different from the control, C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high.

4.3.4 Effect of Sweeteners on GLUT4

The control group had the highest GLUT4 concentration (4.38 ± 0.28 ng/ml) which was significantly higher than all the treatment groups (Table 4.4). ANOVA showed a significant difference in GLUT4 protein concentrations between the different treatment groups [$F(12,45)=13.09$, $p<0.0001$], with a post hoc analysis detecting differences among several groups. Groups with low doses of the sweeteners had higher GLUT4 concentrations than groups administered with medium and high doses.

Low-dose stevia had a significantly lower GLUT4 concentration than the groups on low-dose brown sugar ($p=0.026$) and low-dose honey ($p=0.023$). Medium-dose white sugar had a

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Table 4.4: A One-way Anova and Post Hoc on the Effect of Sweeteners on GLUT4 Concentration

GLUT 4 (ng/ml)	Low Dose Mean± SEM	Medium Dose Mean± SEM	High Dose Mean± SEM	p-value
Control		4.38 ± 0.28		
White Sugar	1.53 ± 0.37[a]	2.94 ± 0.52	2.02 ± 0.27[a]	0.078
Brown Sugar	2.85 ± 0.45[a]	1.72 ± 0.30[a]	0.99 ± 0.05[a]	**0.004***
Honey	2.88 ± 0.39[a]	1.01 ± 0.13[a]	1.31 ± 0.01[a]	**<0.001***
Stevia	1.11 ± 0.13[a]	1.26 ± 0.16[a]	0.33 ± 0.08[a]	**0.002***
p-value	**0.008***	**<0.0001***	**<0.0001***	

Results are presented as means ± SEM, n=6, *values are statistically significant, [a]significantly different from the control, C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high. P value < 0.05 was considered significantly different.

significantly higher GLUT4 concentration than medium-dose honey and stevia (p=0.004, p=0.014 respectively). Within the high-dose group, the experimental group administered with high-dose white sugar had a significantly higher GLUT4 concentration compared to groups on high-dose brown sugar (p=0.001), honey (p=0.030), and stevia (p<0.0001). Similarly, high-dose stevia had a significantly lower GLUT4 concentration than high-dose brown sugar (p=0.045) and high-dose honey (p=0.005). Generally, stevia was observed to cause a reduction in GLUT4 concentration

4.3.5 Effect of Sweeteners on Hexokinase II (HK2) Enzyme

Hexokinase II concentration was highest with the administration of low-dose stevia (200.5 ± 19.4 pg/ml) and highest in the group administered with high-dose white sugar (70.7 ± 5.8 pg/ml) (Table 4.5). ANOVA showed a significant association between treatment groups and HK2 concentration [F (12, 64) = 11.66, *p*=0.0001]. A post hoc analysis showed differences between several pairs. In

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comparing the treatment groups with the control, the group on high-dose white sugar had a significantly lower HK2 concentration than the control (p=0.0004) whereas the group on low-dose stevia had a significantly higher HK2 than the control (p<0.0001) (Table 4.5).

Low-dose stevia group had a significantly higher HK2 concentration than low-dose white sugar (p=0.002), low-dose brown sugar (p=0.007) and low-dose honey (p=0.001). Among the medium-dosage administered groups, medium-dose brown sugar had a significantly higher HK2 concentration than medium-dose honey (p=0.008). High-dose white sugar had a significantly lower HK2 than brown sugar (p=0.045), honey (p=0.019), and stevia (p<0.0001). High-dose stevia had a significantly higher HK2 concentration than high-dose white sugar and honey (p=0.011, p=0.027 respectively) (Table 4.5). Generally, a decrease in HK2 was observed with the administration of high doses of sweeteners.

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Table 4.5: A One-way Anova and Post Hoc on the Effect of Sweeteners on HK2 Concentration

HEXOKINASE (pg/ml)	Low Dose Mean± SEM	Medium Dose Mean± SEM	High Dose Mean± SEM	p-value
Control		128.7 ± 8.9		
White Sugar	129.7 ± 11.4	122.4 ± 6.4[a]	70.7 ± 5.8	**0.0002***
Brown Sugar	137.6 ± 6.0	148.0 ± 4.2	98.1 ± 3.1	**<0.0001***
Honey	119.3 ± 5.2	112.7 ± 5.1	101.9 ± 6.7	0.130
Stevia	200.5 ± 19.4[a]	129.1 ± 10.2	131.7 ± 9.8	**0.003***
p-value	**0.019***	**0.012***	**<0.0001***	

Results are presented as means±SEM, n=6, *values are statistically significant, [a]significantly different from the control, C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high. P value < 0.05 was considered significantly different.

4.4 LIPID PROFILE

Section 4.4 provides a summary of the results on the effect of the sweeteners on the lipid profile (total cholesterol, triglycerides, HDL, LDL, and VLDL) and the lipid ratios (Castelli Risk Index I and II, Atherogenic Plasma Index).

4.4.1 The Effect of Sweeteners on Total Cholesterol (TC)

The control group had the highest total cholesterol concentration (2.38±0.08 mmol/l) which was not significantly different form the lowest level observed in the medium dose honey group (1.93±0.13 mmol/l) (p=0.177) (Figure 4.6).

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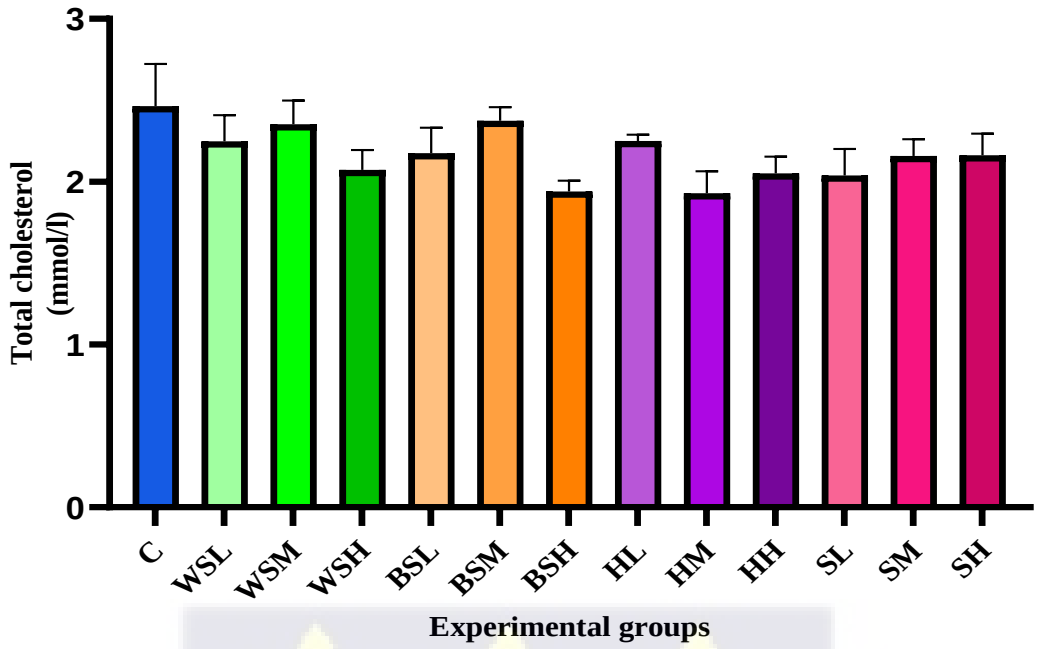

Figure 4.6: Total cholesterol after 17 weeks of treatment. The control group had the highest total cholesterol with the least in medium dose honey. There was no defined trend in the fasting blood glucose at end line in the various groups with exception of stevia. There was a dose dependent increase in total cholesterol with stevia administration. C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high.

4.4.2 The Effect of Sweeteners on Triglyceride Concentration (TG)

Triglyceride level was lowest in the brown sugar low-dose (0.67±0.08 mmol/l) and highest in rats administered with high dose stevia (2.66±0.37 mmol/l) (Table 4.6). The groups administered with stevia had TG levels increasing in a dose-dependent manner. An ANOVA test showed a statistically significant difference in the mean TG concentration among the various treatment groups [F (12.0, 65.0) = 2.9, p=0.003]. A post hoc analysis revealed significant differences among various groups.

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Table 4.6: A One-way Anova and Post Hoc on the Effect of Sweeteners on Triglyceride Concentration

TRIGLYCERIDE (mmol/l	Low Dose Mean± SEM	Medium Dose Mean± SEM	High Dose Mean± SEM	p-value
Control		0.77 ± 0.04		
White Sugar	1.20 ± 0.17[a]	1.16 ± 0.17[a]	1.09 ± 0.17	0.877
Brown Sugar	0.67 ± 0.08	1.48 ± 0.31	1.13 ± 0.24	0.074
Honey	0.94 ± 0.27	2.12 ± 0.16[a]	1.56 ± 0.25	**0.009***
Stevia	1.99 ± 0.07[a]	2.44 ± 0.09[a]	2.66 ± 0.37[a]	0.127
p-value	**0.0001***	**0.0008***	**0.0014***	

Results are presented as means±SEM, n=6, *values are statistically significant, [a]significantly different from the control, C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high. P value < 0.05 was considered significantly different.

When compared with the control group medium dose honey (p=0.021) and all doses of stevia (p<0.0001, p<0.0001, p=0.023, from low to high respectively). Within the high dose group, stevia had a significantly higher TG concentration than white sugar, brown sugar, and honey (p=0.002, p=0.003, p=0.038). Generally, TG was highest within the stevia group.

4.4.3 The effect of Sweeteners on High-Density Lipoprotein Cholesterol (HDL-C)

The group administered with low-dose honey had the highest HDL concentration (1.72 ± 0.19 mmol/l) (Table 4.7). An ANOVA test showed a statistically significant association between high-density lipoprotein and the various treatment groups [F (12, 65) = 2.003, p=0.0380].

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Table 4.7: A One-way Anova and Post Hoc on the Effect of Sweeteners on HDL-C Concentration

HDL-C (mmol/l)	Low Dose Mean± SEM	Medium Dose Mean± SEM	High Dose Mean± SEM	p-value
Control		1.29 ± 0.17		
White Sugar	1.33 ± 0.11	1.34 ± 0.12	1.02 ± 0.16	0.332
Brown Sugar	1.45 ± 0.12	1.54 ± 0.08	1.42 ± 0.07	0.648
Honey	1.72 ± 0.19	1.42 ± 0.05	1.43 ± 0.08	0.169
Stevia	1.43 ± 0.11	1.54 ± 0.09	1.56 ± 0.09	0.594
p-value	0.228	0.351	0.008	

Results are presented as means±SEM, n=6, *values are statistically significant, C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high. P value < 0.05 was considered significantly different.

In pulling all the low doses and medium doses together, there was no significant difference in the mean of their HDL (p=0.228, p=0.351 respectively), however, there was a significant difference in the mean HDL concentration when all the high dosages were pulled together (p=0.008) with white sugar having a significantly lower mean HDL than honey (p=0.045) and stevia (p=0.007). In summary, the greatest reduction in HDL was seen in the administration of high-dose of white sugar.

4.4.4 The Effect of Sweeteners on Low-Density Lipoprotein Cholesterol (LDL-C)

The experimental group administered with medium-dose white sugar had the highest LDL (0.55 ± 0.07 mmol/l) (Table 4.8). A statistically significant association was found between the treatment groups and LDL concentration at the end of the study [F (12, 65) = 3.252, *p*=0.001].

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When compared with the control group, LDL was significantly higher in the control group than the high-dose brown sugar (p=0.012), medium and high dose honey (p=0.012, p=0.022 respectively) and low and high dose stevia (p=0.008, p=0.018 respectively). In pulling all the low doses and high doses together, there was no significant difference in LDL concentration. In the medium dosage groups, white sugar had a significantly higher LDL concentration than honey (0.019).

4.4.5 The Effect of Sweeteners on Very Low-Density Lipoprotein (VLDL)

The highest concentrations of VLDL were in the group administered with high-dose brown sugar (0.68±0.24 mmol/l) (Table 4.9). An ANOVA test revealed a significant association between the type of sweetener and dosage received and VLDL concentration [F (12, 65) = 2.43, p=0.011]. A post hoc analysis showed that the control group had a significantly lower VLDL concentration than the group on high-dose brown sugar (p=0.014).

There was no significant difference in VLDL concentration comparing different dosages of white sugar, brown sugar, and stevia. However, there were significant differences in VLDL concentration among the groups administered with different dosages of honey (p=0.005). The group on medium-dose honey had a significantly higher VLDL concentration than the group on low-dose honey (p=0.004).

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Table 4.8: A One-way Anova and Post Hoc on the Effect of Sweeteners on LDL-c Concentration

HDL-C (mmol/l)	Low Dose Mean± SEM	Medium Dose Mean± SEM	High Dose Mean± SEM	p-value
Control		0.52 ± 0.051		
White Sugar	0.48 ± 0.05	0.55 ± 0.07	0.44 ± 0.04	0.374
Brown Sugar	0.41 ± 0.04	0.49 ± 0.02	0.36 ± 0.03[a]	0.043
Honey	0.44 ± 0.01	0.36 ± 0.03[a]	0.37 ± 0.03[a]	0.084
Stevia	0.35 ± 0.04[a]	0.39 ± 0.03	0.36 ± 0.03[a]	0.567
p-value	0.139	0.017	0.299	

Results are presented as means ± SEM, n=6, *values are statistically significant, [a] significantly different from the control, C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high. P value < 0.05 was considered significantly different.

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Table 4.9: A One-way Anova and Post Hoc on the Effect of Sweeteners on VLDL-c Concentration

VLDL-C (mmol/l)	Low Dose Mean± SEM	Medium Dose Mean± SEM	High Dose Mean± SEM	p-value
Control		0.18 ± 0.02		
White Sugar	0.25 ± 0.03	0.23 ± 0.03	0.23 ± 0.03	0.922
Brown Sugar	0.18 ± 0.02	0.52 ± 0.26	0.68 ± 0.24[a]	0.247
Honey	0.18 ± 0.05	0.45 ± 0.03	0.32 ± 0.05	0.005
Stevia	0.38 ± 0.02	0.48 ± 0.02	0.53 ± 0.07	0.056
p-value	0.001	0.443	0.016	

Results are presented as means (SEM), n=6, *values are statistically significant, [a] significantly different from the control, C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high.

In comparing the groups administered with low dosages of sweeteners, there was a significant difference in VLDL concentration (p=0.0011). The group administered with low-dose stevia had a significantly higher VLDL concentration than the experimental group on low-dose brown sugar (p<0.001) and low-dose honey (p=0.003). Overall, in comparing the treatment groups on high doses of the sweeteners, the group on high-dose stevia had a significantly higher VLDL concentration than high-dose white sugar (p=0.025). There was no significant difference in VLDL concentration among the groups on medium doses of the various sweeteners. Generally, VLDL-c was low with the intake of white sugar compared with the other sweeteners.

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4.4.6 Castelli Risk Index I (CRI-I)

The group administered with high-dose white sugar had the highest CRI-I score (2.30 ± 0.3) (Table 4.10). A significant association was found between the experimental groups ($F(12, 65) = 4.5$, $p<0.0001$), specifically within the high dose group, white sugar had a higher CRI-I than brown sugar, honey, and stevia ($p=0.011$, $p=0.012$, $p=0.011$) (Table 4.10). Thus, administration of high-dose white sugar was observed to increase Castelli Risk Index I.

4.4.7 Castelli Risk Index II

Castelli Risk Index II was highest with high dose of white sugar (0.49 ± 0.09) (Table 4.11) and lowest in high dose stevia (0.23 ± 0.01). CRI-II increased in a dose-dependent manner for white sugar. There was a significant association between CRI-II and the type and dosage of sweetener [$F(12, 65) = 5.82$, $p<0.0001$]. A post hoc analysis showed significant differences in CRI-II between different treatment groups. The control group had a significantly higher CRI-II than the treatment groups on high-dose brown sugar ($p=0.012$), all doses of honey ($p=0.022$, $p=0.012$, $p=0.018$, from low to high respectively) and all doses of stevia ($p=0.006$, $p=0.022$, $p=0.004$, from low to high respectively).

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Table 4.10: A One-way Anova and Post Hoc on the Effect of Sweeteners on CRI-I

CRI-I	Low Dose Mean± SEM	Medium Dose Mean± SEM	High Dose Mean± SEM	p-value
Control		1.86 ± 0.12		
White Sugar	1.74 ± 0.19	1.79 ± 0.11	2.30 ± 0.36	0.229
Brown Sugar	1.51 ± 0.06	1.55 ± 0.05	1.39 ± 0.09	0.219
Honey	1.36 ± 0.09	1.37 ± 0.08	1.44 ± 0.03	0.724
Stevia	1.43 ± 0.02	1.40 ± 0.03	1.39 ± 0.02	0.478
p-value	0.113	**0.0009***	**0.005***	

Results are presented as means (SEM), n=6, *values are statistically significant, [a] significantly different from the control, C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high

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Table 4.11: A One-way Anova and Post Hoc on the Effect of Sweeteners on CRI-II

CRI-II	Low Dose Mean± SEM	Medium Dose Mean± SEM	High Dose Mean± SEM	p-value
Control		0.42 ± 0.02		
White Sugar	0.37 ± 0.05	0.40 ± 0.02	0.49 ± 0.09	0.369
Brown Sugar	0.29 ± 0.03	0.32 ± 0.02	0.25 ± 0.02[a]	0.178
Honey	0.26 ± 0.02[a]	0.25 ± 0.03[a]	0.26 ± 0.02[a]	0.941
Stevia	0.24 ± 0.01[a]	0.26 ± 0.02[a]	0.23 ± 0.01[a]	0.371
p-value	0.052	**0.0001***	**0.0024***	

Results are presented as means (SEM), n=6, *values are statistically significant at p<0.05, [a] significantly different from the control, C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high

There was no significant difference in CRI-II in comparing the treatment groups on low doses of the sweeteners. However, among the medium doses, treatment groups on white sugar had a significantly higher CRI-II than brown sugar (p=0.049), honey (p=0.002) and stevia (p=0.004). A similar observation was made with the treatment groups on high doses, with the group on white sugar having a significantly higher CRI-II than the treatment groups on brown sugar (p=0.009), honey (p=0.041) and stevia (p=0.004). The greatest effect on CRI-II was observed with white sugar administration.

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4.4.8 Atherogenic Index of Plasma

The highest Atherogenic Index of Plasma (AIP) was observed in the group on high-dose stevia (0.21±0.07) (Table 4.12). A one-way ANOVA showed a significant association between treatment groups and AIP [F (12, 65) =5.56, p<0.0001]. A post hoc analysis detected differences in several groups.

The control group had a significantly lower AIP than all the treatment groups on doses of stevia (p=0.035, p=0.009, p=0.008, from low to high respectively) and medium-dose honey (p=0.022). Low-dose stevia was significantly higher than low-dose brown sugar (p=0.001), low-dose honey (p=0.001). AIP increase in the stevia group was in a dose-dependent manner. Generally, stevia was observed to cause an increase in AIP.

Table 4.12: A One-way Anova and Post Hoc on the Effect of Sweeteners on AIP

AIP	Low Dose Mean± SEM	Medium Dose Mean± SEM	High Dose Mean± SEM	p-value
Control		0.18 ± 0.04		
White Sugar	0.39 ± 0.07	0.33 ± 0.05	0.38 ± 0.08	0.349
Brown Sugar	0.20 ± 0.08	0.52 ± 0.18	0.46 ± 0.11	0.127
Honey	0.25 ± 0.12	0.78 ± 0.05[a]	0.61 ± 0.06	**0.002***
Stevia	0.77 ± 0.05[a]	0.79 ± 0.04[a]	0.85 ± 0.06[a]	0.6105
p-value	**0.0003***	0.196	**0.018***	

Results are presented as means (SEM), n=6, *values are statistically significant, [a] significantly different from the control, C=Control, WSL=white sugar low, WSM=white sugar medium, WSH=white sugar high, BSL=brown sugar low, BSM=brown sugar medium, BSH=brown sugar high, HL=honey low, HM=honey medium, HH=honey high, SL=stevia low, SM=stevia medium, SH=stevia high

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4.5 LINEAR REGRESSION OF AIP AS A FUNCTION OF END LINE FASTING PLASMA GLUCOSE, GLUT4, FASTING INSULIN AND HEXOKINASE 2

A multiple regression analysis was performed to predict AIP on the basis of fasting plasma glucose, GLUT4 concentration, fasting insulin, and Hexokinase 2 concentration. The results from the ANOVA table show that the regression model was significant (p = 0.040) (Table 4.13). The model can explain approximately 18% of the variation in AIP (R^2 = 0.18). The GLUT4 concentration was a significant predictor of AIP (p= 0.006), and a unit increase in GLUT4 concentration will lead to a 0.10 reduction in AIP. Other variables such as endpoint FPG, Fasting Insulin, and HK 2 were not related to AIP significantly.

Table 4.13: Multiple regression analysis of AIP as a function of FPG, GLUT4, Fasting Insulin and HK 2

Variables	B	T	Sig	95% CI for B	
				Lower Bound	Upper Bound
End line FPG	0.027	1.069	0.290	-0.023	0.077
GLUT4	-0.101	-2.878	0.006	-0.171	-0.030
Fasting Insulin	-0.009	0.899	0.373	-0.011	0.030
HK 2	0.001	0.620	0.538	-0.002	0.003
Constant	-0.022	-0.148	0.883	-0.321	0.277

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4.6 EFFECT OF THE SWEETENERS ON THE HISTOLOGY OF THE BODY ORGANS

The effect of chronic intake of sweeteners on the histology of the brain, pancreas, and muscles is described in this section.

4.6.1 Effect of Sweeteners on the Histology of the Pancreas

The histopathology of the control rat pancreas showed normal islets of Langerhans with pale rounded and ovoid β-cells (Figure 4.7). Pancreas from rats administered with low and medium doses of white sugar did not show any change in appearance microscopically. However, a representative section of the pancreas from a rat receiving a high dose of white sugar showed overstretched pancreatic ducts (Figure 4.8a) and perivascular infiltration of leucocytes (Figure 4.8b).

Similarly, pancreas from rats administered with a low-dose and medium-dose of brown sugar showed no histological changes. On the other hand, the pancreas from a rat administered with high-dose of brown sugar showed haemorrhages in the interlobular spaces (Figure 4.9a) and perivascular oedema (Figure 4.9b).

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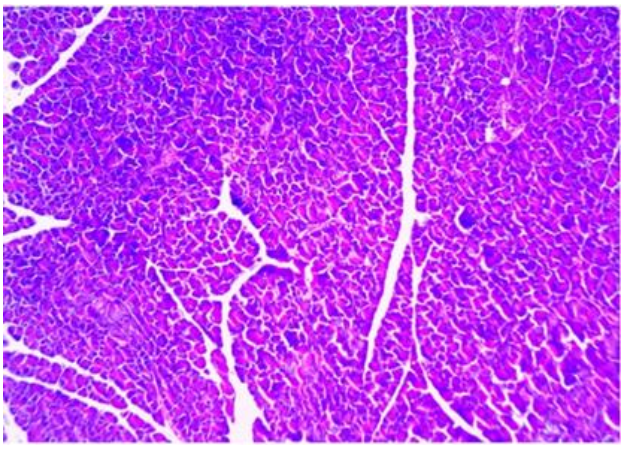

Figure 4.7: Pancreas of control rat with normal islets of Langerhans, with pale rounded and ovoid β cells.

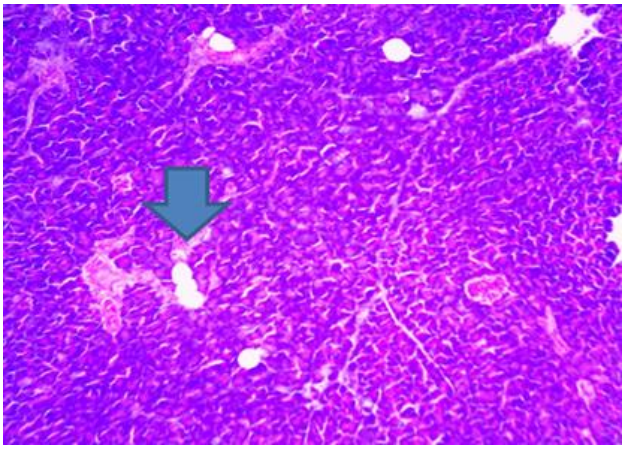

Figure 4.8a: Pancreas of high-dose white sugar, showing over stretched pancreatic ducts (arrow).

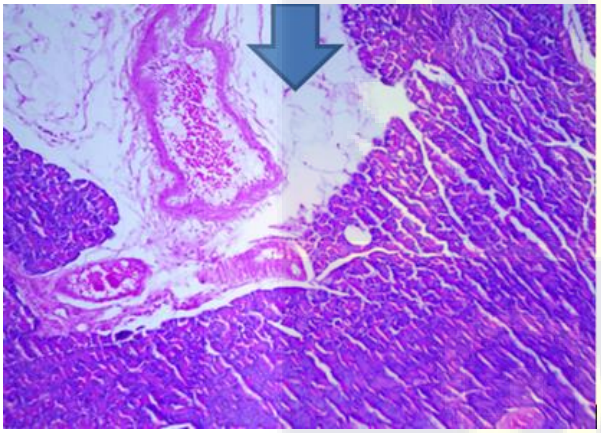

Figure 4.8b: Pancreas of rat with high dose white sugar, showing Leucocytes infiltration (arrow).

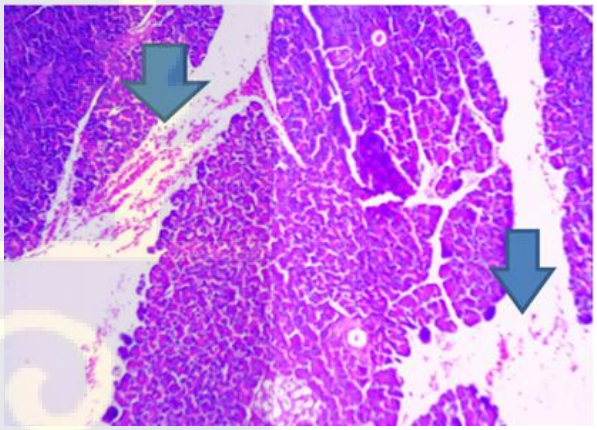

Figure 4.9a: Pancreas of high dose brown sugar, showing haemorrahage in interlobular spaces (arrows)

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Also, rats administered with low dose honey showed normal pancreatic structure with no change histopathologically. Although the pancreas from medium-dose honey showed no appreciable change under the microscope, the pancreatic ducts appeared overstretched (Figure 4.10). Rats receiving high dose honey also showed no appreciable changes histologically. Among the stevia administered groups, only pancreas from the medium-dose stevia showed histological changes. Medium-dose stevia showed slight congestion in interlobular spaces (Figure 4.11).

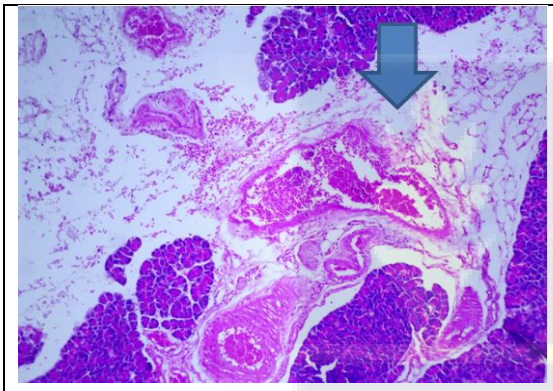

Figure 4.9b: Pancreas of high dose brown sugar, showing perivascular oedema (arrow).

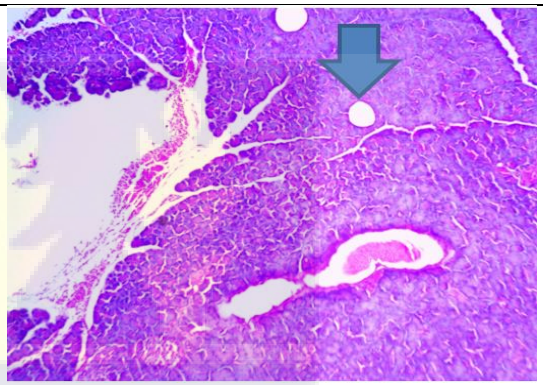

Figure 4.10: Pancreas of medium dose honey, arrowing showing overstretched pancreatic ducts (arrow).

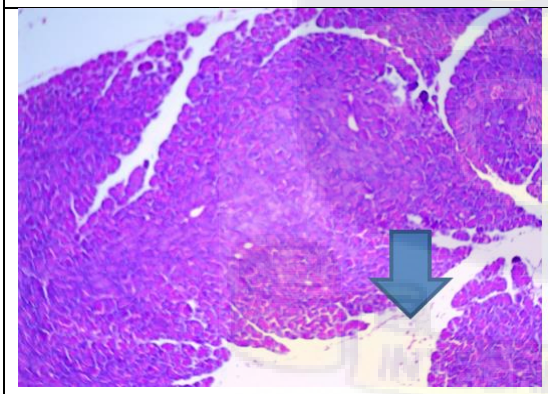

Figure 4.11: Pancreas of medium dose stevia showing slight congestion in interlobular space (arrow).

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4.6.2 Effect of Sweeteners on the Histology of the Skeletal Muscle

A longitudinal section through the skeletal muscle of the control rat showed adequately preserved myofibers with clear striation and peripheral myonuclei (Figure 4.12)**.** Sections of the skeletal muscle of rats administered with white sugar, brown sugar and honey showed no pathological changes microscopically. Effect of the sweeteners on the skeletal muscle was observed with stevia administration.

Sections through the skeletal muscle of rats administered with low-dose rat showed altered muscle fibres which appeared enlarged at various extents, distinctly eosinophilic with absent nuclei (Figure 4.13a). The low dose stevia also showed degenerative necrobiotic changes from the Zenker's necrosis type with enhanced acidophilia (Figure 4.13b). The medium dose stevia also showed degenerative changes in the longitudinal muscle fibres (Figure 4.14a). Longitudinal sections of the skeletal muscle of rats administered with high dose stevia showed areas of inflammation infiltrating the fibres of muscles (Figure 4.14b). The affected zone showed an increase in the number of inflammatory cells invading the intercellular spaces. Occasionally, small areas of necrosis and lyses were seen among normal looking fibres.

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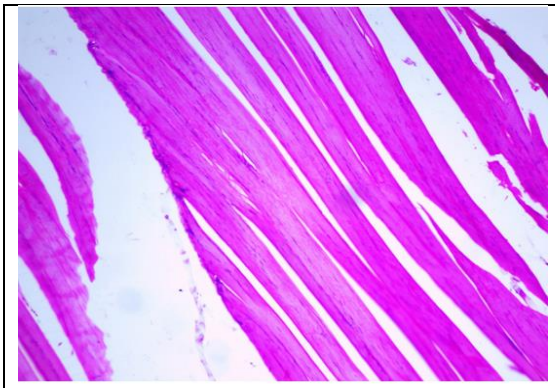

Figure 4.12: Skeletal muscle of control rat, adequately preserved myofibers, clear striation

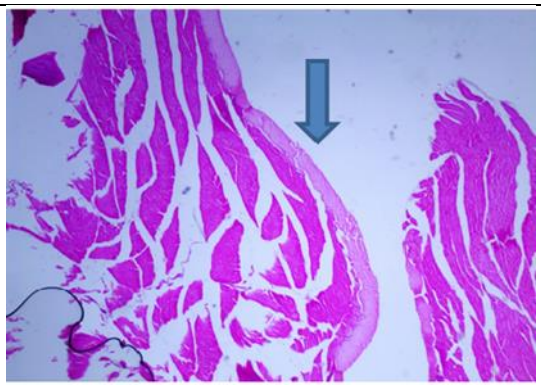

Figure 4.13a: Skeletal muscle of low-dose stevia showing altered muscle fibers enlarged at various extents (arrow).

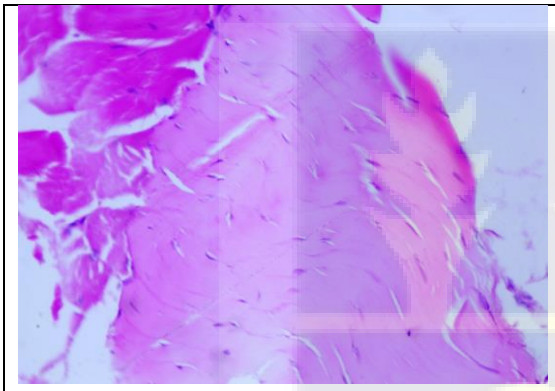

Figure 4.13b: Skeletal muscle of low-dose stevia, degenerative necrobiotic changes

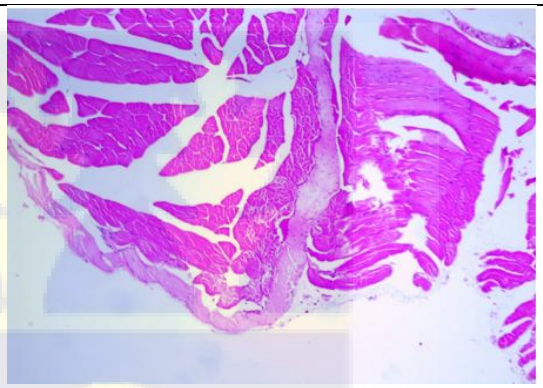

Figure 4.14a: Skeletal muscle of medium-dose stevia, degenerative changes

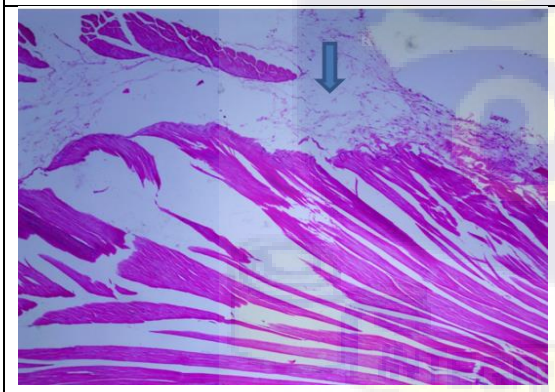

Figure 4.14b: Skeletal muscle of high-dose stevia showing areas of inflammation infiltrating muscle fibers with small areas of necrosis and lysis (arrow).

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4.6.3 Effects of Sweeteners on the Histology of the Brain

The main effect of the sweeteners on the brain was on the hippocampus. The control rat had normal hippocampal formation. The hippocampus of rats administered with low, medium, and high doses of white sugar showed a normal appearance (Figure 4.15a). However, there was widespread infiltration of glial cells in the cerebral cortex of the brain in the medium-dose white sugar rat (Figure 4.15b) and a few infiltrations of reactive cells in the CA3 area of the hippocampus in the high-dose rat (Figure 4.15c).

Brain from a rat administered with low-dose brown sugar had a normal appearance of the hippocampus. However, medium-dose brown sugar showed marked congestion in the cerebral region (Figure 4.16a). A representative section of the brain from rats that were administered with a high dose of brown sugar showed vacuolation into the deep parenchymal tissues of the cerebrum (Figure 4.16b). There was also widespread death of neuronal cells (Figure 4.16b). Brain from high doses of brown sugar also showed marked variation in the thickness of the hippocampus area and infiltrations of mononuclear cells along with haemorrhage in the epidural space (Figure 4.16c).

Among the stevia-administered group, a representative section of the brain from rats administered with medium-dose stevia showed vascular dilatation and congestion on the pia matter with infiltrations of reactive cells (Figure 4.17a). There was also sub-arachnoid haemorrahage with vacuolations found on the peripheral brain parenchyma (Figure 4.17a). A representative section of the brain of rats administered with high-dose stevia showed cerebral oedema in the hippocampus indicated by an increase in perivascular spaces (Figure 4.17b).

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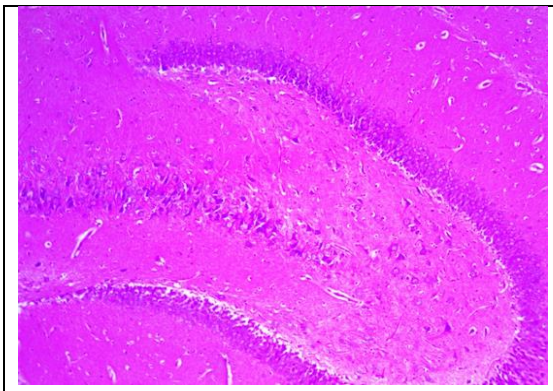

Figure 4.15a: Hippocampus of low-dose white sugar, normal hippocampal formation

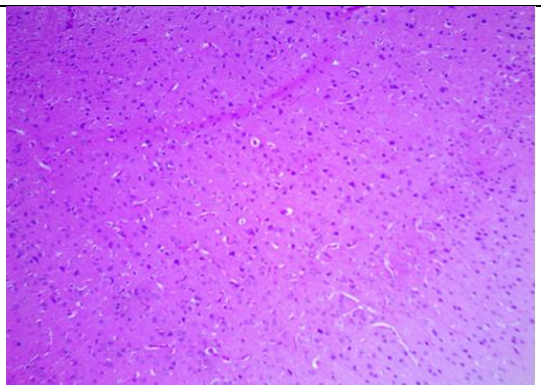

Figure 4.15b: Cerebral cortex of medium-dose white sugar, infiltration of glial cells in the cerebral cortex

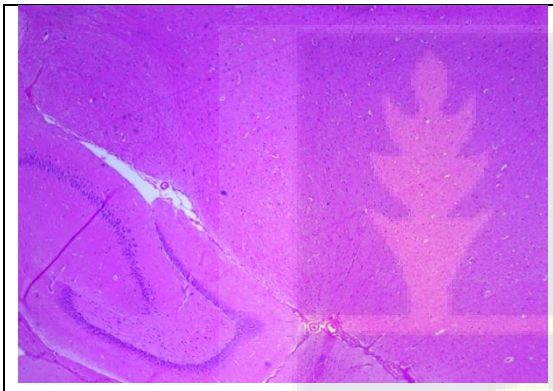

Figure 4.15c: Hippocampus of high dose white sugar, infiltration of reactive cells in the CA3 area of the hippocampus.

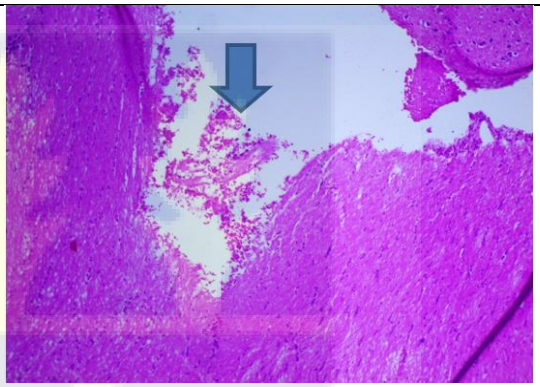

Figure 4.16a: Cerebral cortex of medium dose brown sugar, arrow showing marked congestion in the cerebral region.

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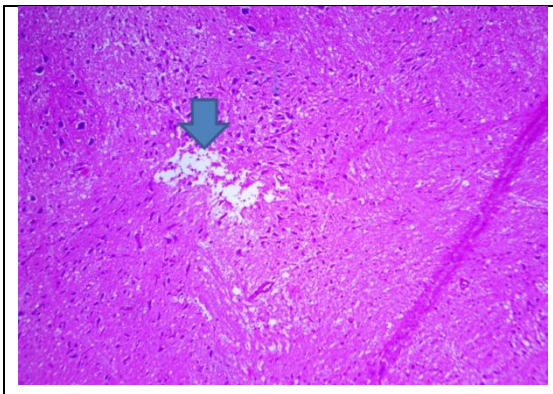

Figure 4.16b: Cerebrum of high dose brown sugar, arrow showing widespread death of neuronal cells.

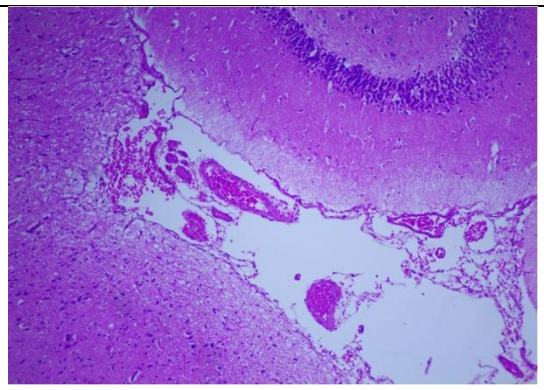

Figure 4.16c: hippocampus of high dose brown sugar, marked variation in the thickness of hippocampal area, infiltration of mononuclear cells, haemorrahage in epidural space.

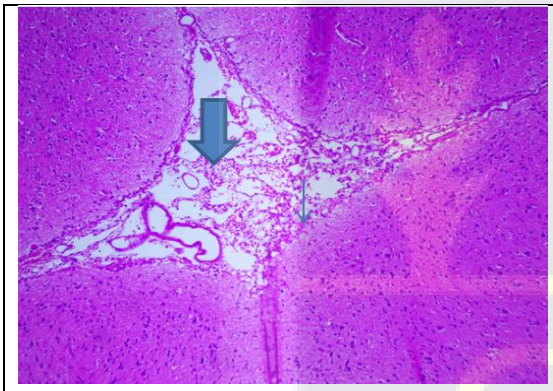

Figure 4.17a: Brain of high-dose stevia, arrow showing vascular dilatation, infiltration of reactive cells, congestion of the pia matter, subarachnoid haemorrahage on peripheral brain parenchyma

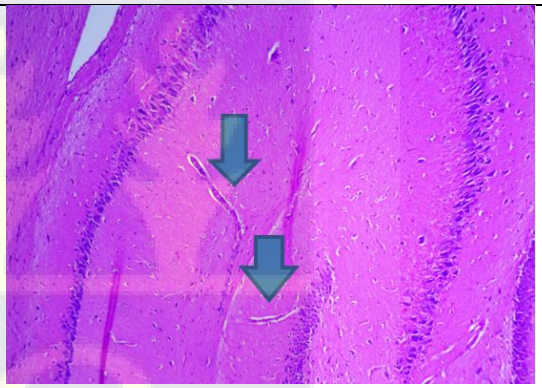

Figure 4.17b: Hippocampus of high dose stevia, arrow showing oedema in the hippocampus

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CHAPTER 5

5.0 DISCUSSIONS

5.1 SWEETENERS AND HEALTH

The increase in the production and intake of caloric sweeteners has been seen paralleling the rise in metabolic syndrome. Due to health-related effects attributed to the intake of caloric sweeteners, there is an increase in the production and intake of LNCS (Stanhope, 2016a). However, there is mounting evidence that indicates that LNCS may also contribute to the development of metabolic syndrome or its individual components (obesity, insulin resistance or type 2 diabetes, high TG, low HDL, high blood pressure) although they do not contribute to caloric intake (Singh et al., 2023). Low or No Calorie Sweeteners have been shown to modulate intestinal bacteria and also reduce postprandial leptin release increasing food intake which could contribute to weight gain (Khan et al., 2020). Caloric sweeteners provide excess calories contributing to weight gain or excess fat deposition, a risk factor for insulin resistance. Insulin resistance as a criterion in the diagnosis of metabolic syndrome is also a risk factor for the other components of metabolic syndrome: type 2 diabetes, dyslipidemia, and hypertension. Preventing and controlling NCDs, such as metabolic syndrome is imperative in achieving the sustainable development goal. Cardiovascular diseases and diabetes are included in the four main causes of mortality worldwide with diabetes-related mortality increasing by 3% between 2000 and 2019 despite the decline in cardiovascular disease mortality (WHO, 2023b).

To promote healthy eating, WHO recommended that daily intake of added sugars should be less than 10% of daily energy intake or decreasing it further to 5% of the total energy intake for the day (World Health Organisation, 2018). This recommendation assumes that all sweeteners have

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the same effect on the body without considering the carbohydrate composition of each sweetener and the varying effect they may have on health. Experimental studies have explored the relationship between the intake of sweeteners (both caloric and non-caloric) on the various indices of metabolic syndrome including insulin resistance without examining possible mechanisms involved (Abo Elnaga et al., 2016; Barrios-Correa et al., 2018; Bigos et al., 2012; Figlewicz et al., 2009; Farid et al., 2020; Khamis, Tayel, & Aborhyem, 2020; Nwachuku et al., 2017; Rosales-gómez et al., 2018; Samat, Kanyan Enchang, Nor Hussein, & Wan Ismail, 2017). This study sought to explore the relationship between different types and doses of sweeteners on metabolic health and also the underlying mechanism.

5.2 EFFECT OF SWEETENER INTAKE ON ENERGY INTAKE

The highest energy intake from rat chow was observed in rats being administered with high-dose of white sugar (461.9 Kcal) followed by high dose of stevia (421.1 kcal). Food behaviours which includes meal preferences, timing and quantity of consumption have been shown to be regulated by appetite regulating centers in the hypothalamus (Hyun Choi et al., 2022). Added sugars, particularly fructose down regulates satiety centers in the hypothalamus which stimulates hunger causing increased food intake (Hyun Choi et al., 2022; May, Rosander, Gottfried, Dennis, & Dus, 2020; Penaforte, Japur, Pigatto, Chiarello, & Diez-Garcia, 2013). The presence of fructose from the white sugar could account for the increased food consumption observed in this study by causing an increase in serum ghrelin which stimulates hunger and whilst suppressing satiety centers of the brain (Lowette et al., 2015).

In a study among sixteen healthy women, a high intake of sugar during breakfast was observed to be followed by increased pre-prandial hunger causing an increase in food intake during lunch time (Penaforte et al., 2013). Monteiro-Alfredo et al. (2021a) in their study provided administered

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sugary solution *ad libitum* for 4 weeks to Goto-kakizaki rats. This impaired their energy balance and caused an increased food intake and weight gain compared to rats consuming fruit juices *ad libitum*. Fruit juices, contain natural sugars in addition to other nutrients such as fibre which delays gastric emptying hence regulating the absorption of the sugars into the blood stream and its side effects (Monteiro-Alfredo et al., 2021b). These support the theory that consumption of refined or added sugars such as white sugar influences satiety and consequently increases food intake as observed in this study.

There remains conflicting evidence on the intake of LNCS on food intake and energy compensation. Research evidence suggests that LNCS may increase appetite with a corresponding energy intake and increase in weight gain (Mattes & Popkin, 2009). It has been shown that the consumption of sweeteners activates sweet taste receptors on the tongue, triggering the cephalic phase of digestion with the body expecting the provision of calories from the sugars. The cephalic phase triggers a series of events which culminate in initiating satiety. However, in the case of LNCS, since they do not provide calories, their consumption impairs the cephalic phase. Hence an increase in food intake, and subsequent weight gain is inevitable (Shum & Georgia, 2021b).

In a short-term study, rats administered with saccharin solution were observed to eat more chow in comparison with those administered with only water (Tordoff, 1988). In another study by Swithers & Davidson (2008), the authors tested this hypothesis in male Sprague Dawley rats. They observed that rats administered with saccharin sweetened yoghurt had a higher energy intake compared with rats given glucose sweetened yoghurt. However, the evidence remains inconclusive regarding the role of LNCS on caloric intake (Shum & Georgia, 2021b). Farhat, Berset, & Moore, (2019) in their study examined the effect of stevia extract, one of the least experimented sweeteners on appetite and food consumption among individuals who are healthy. Stevia was reported to lower

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appetite sensation and food intake. Similarly, in a double-blind randomized control trial among healthy individuals, consumption of a beverage containing stevia before lunch decreased appetite and subsequently energy intake during lunch (Stamataki et al., 2020). However, these trials were short term, hence may not be a true reflection of energy compensation in the long term (Mattes & Popkin, 2009).

5.3 EFFECT OF SWEETENER INTAKE ON PERCENT WEIGHT GAIN AND FOOD EFFICIENCY RATIO

In this present study, the greatest effect of the sweeteners on percent weight gain was observed with high-dose stevia administration, followed by low-dose honey and high-dose white sugar administration. The least weight gain was observed in the control group. Epidemiological and experimental studies have suggested that although sweeteners cause weight or obesity, the dose consumed is what makes them more harmful (Atangwho et al., 2020a; Gillespie, Kemps, White, & Bartlett, 2023a; Stanhope, 2016a).

In this present study, there was a dose dependent increase in weight gain with the intake of white sugar. Diets high in refined sugars have been shown to increase fat deposition, causing obesity (Langin & Viguerie, 2020). Shamsi-Goushki et al. (2021c) in an experimental study reported that 15% of white sugar administered to male Wistar rats for 42 days caused a significant increase in body weight. Similar findings were reported among mice administered with 30% sucrose solution after 12 weeks of administration (Burke et al., 2018). These findings agree with observations made in this current study.

In children and adolescents, added sugars contributed by sugary beverages has been reported to cause weight gain and obesity (Yu et al., 2022). Sugar sweetened beverages have free sugars added

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to them during the processing stage. Free sugars are either in the form of fructose corn syrup, sucrose or fruit juice concentrates (Sousa et al., 2020). White sugar is basically made up of sucrose which is further broken-down during digestion to glucose and fructose molecules. Glucose stimulates insulin release which facilitates the conversion of excess glucose storage in the liver and muscles as glycogen and stored as fat in the liver, muscles and adipose tissue. Fructose has been linked to obesity indirectly by causing overeating by suppressing post prandial ghrelin secretion and causing leptin resistance leading to increased caloric intake and contributing to weight gain (Johnson et al., 2017; Shapiro et al., 2008). Not only does fructose suppress satiety, but it also increases the palatability of food causing overeating and a consequent weight gain. Fructose has also been reported to promote the storage of fat by increasing circulating triglycerides, visceral fat stores, reducing fat oxidation and the production of energy from food stores (Shapiro et al., 2008).

Atangwho et al. (2020a) investigated the effect of honey and granulated white sugar incorporated diets at dosages of 8% and 16% on obesity and inflammatory markers after twenty-nine weeks of administration. Both table sugar and honey administration were associated with significant increase in weight gain compared to the control group. However, the effect on weight gain was pronounce in the honey administered group than the table sugar group. The findings by (Atangwho et al., 2020a) corroborates with the results observed in this study to an extent. In this current study, interestingly, rats on low doses of honey had a higher percent weight gain than rats on all doses of white sugar, and honey. The weight gain observed with intake of low-dose honey in this study contradicts findings of previous studies which had suggested that Honey could potentially have a smaller impact on weight gain in comparison with other sweeteners (Carmody et al., 2007; L. Chepulis & Starkey, 2008b; Nemoseck et al., 2011). A systematic review on the role of honey in

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obesity management showed an anti-obesity effect with honey intake (Ugusman et al., 2022) with most of the studies being animal studies which suggests that the results should be interpreted with caution. However, in this current study, rats administered moderate and high dose honey were not significantly different from the control group which had the least weight gain. This broadly supports previous findings associating honey with less obesogenic effects. It should also be noted that, for most of the clinical trials and animal studies involving honey, confounding factors such as physical activity and overall dietary intake might not have been considered (Ugusman et al., 2022). To gain a better understanding of the role of honey in weight management, a high-quality randomized control trial in humans should be conducted.

To reduce the incidence of obesity associated with the intake of nutritive sweeteners, LNCS have been introduced in the food and manufacturing industry. Recent studies have linked the consumption of LNCS with an increase in weight (Shum & Georgia, 2021a; Walbolt & Koh, 2020b). Several mechanisms have been proposed linking LNCS to weight gain. It has been suggested that LNCS activate the sweet taste receptors which induces a hyperinsulinemic state causing excess fat deposition exacerbating the progression of insulin resistance. Also, because LNCS are not digested, they end up in the colon where they act on colonic bacteria, thereby inducing gut dysbiosis and generating metabolites responsible for insulin resistance and consequently promoting weight gain. Another plausible explanation is the satiety lowering effect of the LNCS, resulting in excess food intake contributing to weight gain (Shum & Georgia, 2021c).

In the current study, stevia had the higher percent weight gain which supports the findings in previous studies. Current recommendations from the WHO emphasizes on a reduction in the intake of LNCS or any other sugar substitutes. According to WHO, LNCS are not safe substitutes for caloric or nutritive sweeteners for weight management in the long term (WHO, 2023a). Sweetened

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beverages or drinks remain a major source of LNCS with LNCS accounting for 4% to 18% of carbonated beverage intake in children and adolescents in the US (Gurina & Simms, 2023; Sylvetsky, Rother, & Brown, 2011). Although snacks have the ability to contribute to energy and nutrient intake in children and adolescents, sweetened beverages or drinks are commonly consumed among children and adolescents (Harris et al., 2023). Taking into consideration the evidence against LNCS in weight management, it will be advisable for individuals to opt for naturally occurring sugars such as fruits unsweetened beverages or cereals (WHO, 2023a). The food efficiency ratio, is a feed utilization parameter which gives an estimation of the body weight gain per gram of chow consumed (Ferreira et al., 2018). The pattern of weight gain seen in the study animals corresponds with the FER.

5.4 EFFECT OF SWEETENERS ON FASTING PLASMA GLUCOSE

Previous studies have associated added sugars in sugar sweetened beverages with impaired glucose tolerance (Drouin-Chartier et al., 2019; Malik & Hu, 2022b). It was hypothesized in this study that, after 17 weeks of administration with sweeteners, the fasting plasma glucose in the treatment group would increase in comparison with the control group. Despite the fact that the results of the study showed significant differences in fasting plasma glucose concentrations at the endpoint, values were not greater than the physiological range for fasting plasma glucose (3.6 mmol – 5.6 mmol) (Riley, 2023). An occasional transient increase in fasting plasma glucose over the 17-week period was observed in the treatment group on medium dose white sugar recording the highest fasting plasma glucose level at the end line. The current investigation also showed that, in comparing the trend in fasting plasma glucose among the nutritive sweeteners, rats on white sugar and brown sugar had slight increases in the fasting plasma glucose as opposed to the slight decrease observed with the administration of honey.

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Current literature from both observational, interventional and systematic reviews studies does not provide enough evidence to demonstrate a clear cause-and-effect correlation between the intake of simple sugars or added sugars on glucose control (Veit et al., 2022b). The relationship could be attributed to their caloric addition to meals, having a lesser effect on satiety resulting in increased energy intake and increased adiposity which are risk factors for developing insulin resistance or impaired glucose tolerance (Veit et al., 2022b). This could explain the findings observed in the present study. This concords with the findings of Atangwho et al. (2020) who found occasional transient differences in fasting blood glucose after 13 weeks of supplementation with diet sweetened honey and table sugar. Similarly, sucrose at a dose of 5.2 mg/ml administered to male and female mice caused a significant reduction in the glycated hemoglobin after 8 and 16 weeks of administration (Farid et al., 2020). This also reflects findings from Monteiro-Alfredo et al. (2021b) who compared the effect of four different natural fruit juices with a matched sugary solution. After 4 weeks of administration the matched sugary solution increased glycemia whereas the natural fruit juices had no effect on both glycemia and weight gain. CDI mice administered sucrose had a slightly elevated glucose level in comparison with the control although not statistically significant (Rosales-gómez et al., 2018).

Recent findings suggests that honey possesses hypoglycemic properties. A systematic review of pre-clinical, clinical, human and animal model studies on the effect of honey on diabetes showed that honey decreased the fasting plasma glucose (Meo et al., 2017). Erejuwa et al. (2016a) examined the effect of honey on glycemia in diabetes induced rats. Honey was administered at dosages of 1.0, 2.0 and 3.0 g/kg over a period of 3 weeks. Low dose (1 g/ kg) and medium dose (2 g/kg) had a reducing effect on fasting plasma glucose. In an earlier study by the same author, 4 weeks of administration of honey to streptozotocin diabetes induced rats resulted in a

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hypoglycemic effect (Erejuwa et al., 2010). Similarly, in alloxan induced diabetic rats, 3-weeks of administration with honey also resulted in a decrease in blood sugar (Fasanmade & Alabi, 2008). Despite the observed decrease seen in the diabetic rats, the normal control rats did not experience a decrease in glucose level.

Honey consists of glucose, fructose, maltose, sucrose and other oligosaccharides with a glycemic index similar to that of glucose. It is therefore expected that honey should cause a rise in blood sugar. However, the exact mechanism underpinning the hypoglycemic effect of honey is not well understood. It has been proposed that it improves the function, growth and survival of the beta cells, attenuates glucotoxicity and increases the anti-oxidant defenses (Erejuwa et al., 2012). Erejuwa et al. (2011a), had earlier postulated that oligosaccharides found in honey could be responsible for its hypoglycemic effect. Contrarily, Samat et al. (2014) observed a significant rise in fasting plasma glucose in comparison with the control after an acute administration of 2000mg/kg body weight of honey in healthy male Sprague Dawley rats. Chepulis, (2007) reported similar findings after 6 weeks of a honey-based diet on glycated hemoglobin. The majority of the studies reporting a very significant decrease in glycemia with honey administration were conducted with diabetic individuals or animal models with diabetes. In this pathological state, honey was reported to improve beta cell recovery, function and or death (Erejuwa et al., 2010; Sirisha et al., 2021). This can partially explain the findings in this study since healthy Sprague Dawley rats were used as opposed to the diabetes induced rats used in previous experiments.

Interestingly, the stevia groups experienced a gradual increase in the fasting plasma glucose, although the values were within the normal range. Stevia is one of the least studied LNCS, which contains no carbohydrate. Carbohydrates are the major source of glucose for the body. Hence it is expected that, since stevia does not contain carbohydrate, it should cause a reduction in glycemia

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instead of the gradual increase observed in this present study. For instance, in a cross over trial by Farhat et al. (2019) involving 30 healthy individuals, stevia sweetener administered for 3 days did not have any effect on post prandial glucose compared with sucrose. Similar findings were reported by Stamataki et al. (2020) after administering a stevia sweetened beverage to healthy individuals over 5 visits. Stevia extract tea administered to type 2 diabetes patients over a period of three weeks did not significantly increase in metabolic parameters including fasting plasma glucose and glycated hemoglobin (Ajami, Seyfi, Abdollah Pouri Hosseini, et al., 2020). However, findings from these previous studies differ from Farid et al. (2020) who reported a significantly elevated glycated hemoglobin levels compared to control group in both acute and long-term stevia sweetener administration in healthy male and female mice. Similarly, 12 weeks of administration of stevia in mice resulted in an increase in glycaemia (Rosales-gómez et al., 2018). This reported hazardous effect of stevia is similar to the findings in this current study. The differences in the effect of stevia reported in the different studies could be as a result of differences in the physiological conditions between healthy animal models and the diabetic ones.

5.5 EFFECT OF SWEETENERS ON FASTING INSULIN AND HOMA-IR

Insulin produced by the pancreatic beta cells facilitates the movement of glucose from the blood into the body organs (muscle, brain, liver and adipose tissue) (Cerf, 2013). From this current study, fasting insulin concentration was observed to decrease significantly with the administration of sweeteners in the treatment groups whereas adequate insulin production was observed in the control group. Insufficient insulin production is a characteristic in the development of insulin resistant type 2 diabetes (Jameson et al., 2015). This insulin insufficiency could result from a reduction in beta cell output, its cell mass or both (Cantley & Ashcroft, 2015). Quantification of the islet cell volume in post-mortem pancreas from type 2 diabetes diagnosed individuals showed

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about a 30% decline in islet cell mass which could be due to glucotoxicity or lipotoxicity (Clark et al., 2001).

According to Paulo et al. (2023), daily consumption of reducing sugars, such as fructose and glucose has the ability to suppress the insulin gene and induce oxidative stress causing beta cell death. Similarly, Ebrahimi et al. (2020) in an animal model demonstrated that, mild increases in blood glucose concentration leads to significant changes in the insulin gene expression. Therefore, the implication of this current study's finding is that, the long-term administration of white sugar, brown sugar, honey and stevia could have caused loss of islet cell mass or altered the insulin gene expression thereby suppressing insulin production in the study population. Based on the results of this study, similar dosages from different sweeteners had different effects on insulin concentration which supports the findings of Paulo et al. (2023) that the impact of sugars or sweeteners on beta cell function is dependent on the type and amount of sweetener being consumed. Also based on the findings of this study, the highest concentration of fasting insulin among the sweetener administered groups were observed with the intake of white and brown sugar. Shamsi-Goushki et al. (2021a) in their study administered white and brown sugar to male Wistar rats for 42 days. Although serum fasting insulin concentration increased with both brown and white sugar, there was a significantly greater increase in the white sugar administered group than the brown sugar. According to Shamsi-Goushki et al. (2021a) the sweetener administered groups had a significantly higher insulin concentration than the control group as opposed to the findings of this study. Similarly, 8 weeks of consumption of fructose in diet-induced obese rats resulted in a significantly higher fasting insulin concentration in comparison with the control (Valle et al., 2020b). Based on the results of Shamsi-Goushki et al. (2021a) and Valle et al. (2020b) the reported fasting insulin concentrations in the sweetener administered groups were elevated than the recommended healthy

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level (5 – 12 uU/ml), however in this current study, the fasting insulin concentration were below the recommended concentration.

The HOMA-IR is a surrogate measure in assessing insulin resistance. In this present study, the HOMA-IR had a trend similar to that of the fasting insulin, where there was decrease in HOMA-IR in the sweetener administered group in comparison with the control group. This result was unexpected based on findings from previous studies where chronic consumption of sweeteners resulted in an increase in HOMA-IR which is suggestive of insulin resistance. For instance, 15 weeks administration of stevia in mice resulted in a significantly higher HOMA-IR, compared with the control and other experimental groups (Rosales-gómez et al., 2018). This is suggestive of reduced insulin sensitivity in mice following long term consumption of stevia. Similarly, Valle et al. (2020b) reported higher HOMA-IR index with chronic consumption of refined sugars as opposed to natural sugars. Findings from Shamsi-Goushki et al. (2021b) corroborates with these studies whereby 42 days administration of white sugar and brown sugar in albino rats resulted in increased HOMA-IR compared with rats in the control group.

The discrepancy in HOMA-IR observed in this study could be attributed to beta cell destruction induced by the chronic intake of sweeteners, thereby reducing insulin secretion. HOMA-IR on the other hand is a measurement of the ability of the beta cells to produce, store, and release insulin and not a marker in assessing beta cell loss which may be the case in this current study. Hence, HOMA IR is only valid when the concentration of insulin in the serum reflects the insulin sensitivity and not secretion. It may also not be an accurate measurement of the total body insulin resistance (Reaven, 2013).

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5.6 EFFECT OF SWEETENERS ON GLUT4 AND HEXOKINASE 2

After 17 weeks of administration with different types and dosages of sweeteners, GLUT4 concentration was observed to be significantly lower in the treatment groups compared with the control. The decrease in GLUT4 concentration could be attributed to the reduced serum insulin concentration in the sweetener groups which could imply whole body insulin resistance. GLUT4 is an insulin dependent glucose transporter responsible for glucose uptake in the skeletal muscle and adipose tissue (Reaven, 2013, Vargas & Carrillo Sepulveda, 2019). In response to a high glucose concentration, insulin from the pancreas attaches to an insulin receptor on the skeletal muscle and adipose tissue. This triggers a chain of chemical reactions culminating in the release of GLUT4 transport protein to the surface of the cell membrane permitting glucose entry into the cytoplasm (Bryant & Gould, 2020). A reduction in insulin-stimulated GLUT4 protein concentration has been reported in mice with metabolic syndrome (Leguisamo et al., 2012b).

Findings from this current study imply that, chronic consumption of sweeteners influences the GLUT4 concentration and ultimately on glucose homeostasis. These finding agrees with the observation of (Benetti et al., 2013) who observed a reduction in GLUT4 expression in the skeletal muscle after 30 weeks of administration of high fructose corn syrup in mice. Although studies (Abo Elnaga et al., 2016; Rosales-gómez et al., 2018; Samat et al., 2014; Shamsi-Goushki et al., 2021b; Valle et al., 2020b) have investigated the impact of sweeteners on insulin resistance, there is a paucity of studies examining their effect on the insulin signaling pathway, particularly on GLUT4 expression on concentration. Consistent with literature, this current study found that, high dosages of sweeteners resulted in significant reduction in GLUT4 concentration especially with brown sugar and stevia. High intake of added sugars have been associated with disease conditions

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such as obesity, diabetes and cardiovascular diseases (Rippe & Angelopoulos, 2016; (Macdonald, 2016b; Yu et al., 2022; Alam, Kim, & Jang, 2022).

It was further observed that the effect on GLUT4 concentration was more pronounced with the administration of stevia in the current study. In grouping the dosage levels, stevia had the lowest GLUT4 concentration. This finding was unexpected and suggests that commercial stevia sweetener has detrimental effects on insulin signaling and insulin resistance which is a key risk factor in the development of the various components of metabolic syndrome (Zhao et al., 2023). This outcome contradicts the findings of Harris et al. (2023) who observed that steviol and stevioside compounds improved insulin resistance in an adipocyte model by up-regulating the GLUT4 mRNA expression. This difference may be attributed to the type of stevia product used. Harris et al. (2023) used steviol and stevioside compounds whereas a highly purified stevia product commercially sold was used in the present study. Highly purified stevia products have purified stevia compounds (Rebaudioside A) blended with erythritol which is also a low-calorie sweetener as opposed to the very pure stevia compounds which contains about 95% of the steviol glycoside (Ashwell, 2015).

Another research objective in the present study was to assess the effect of long-term intake of sweeteners on HK 2. Hexokinase 2 is the most common hexokinase isoform found in insulin sensitive cells like the adipocytes, cardiomyocytes and skeletal muscle cells (Roberts & Miyamoto, 2015b). It catalyses the first step in glucose metabolism by phosphorylating glucose to glucose-6-phosphate. Although significant differences were observed among the different experimental groups, it was generally observed that high doses of nutritive sweeteners resulted in a decrease in HK 2 concentration. This may be indicative of a diet induced loss of muscle HK 2. There are similarities however between the findings of this study and that of Shimobayashi et al. (2023) who

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reported diminished HK 2 expression after mice were fed with a high-fat diet. These findings may suggest a possible effect of specific dietary components on HK 2 expression or activity. A reduction in HK 2 expression or activity will cause a reduction in glucose removal hence an imbalance in glucose homeostasis which may contribute to the development of glucose intolerance (Shimobayashi et al., 2023).

Recently several therapeutic treatments such as cancer and rheumatoid arthritis have targeted HK 2. However, there is a paucity of literature on its role in the development of metabolic syndrome or cardiovascular disease. To the best of my knowledge no study has been conducted to assess the effect of chronic consumption of nutritive sweeteners and LNCS on HK 2 expression and concentration, hence making it difficult to interpret these findings in the light of other literature.

5.7 EFFECT OF SWEETENERS ON LIPID PROFILE

According to WHO, CVD remains the leading cause of mortality globally (World Health Organization, 2020). Dyslipidemia has long been identified as a risk factor in cardiovascular conditions (Hedayatnia et al., 2020). Excessive consumption of dietary carbohydrates, particularly added sugars have been identified as a modifiable risk factor in the development of dyslipidemia or an imbalance in the lipid profile (Welsh et al., 2010b). This current study also set out to assess the effect of added sweeteners on the lipid profile in the experimental animal model.

Total blood cholesterol is the sum of both “good” and “bad” cholesterol deposits in the blood. There was a lack of significant differences in the total cholesterol after 17 weeks of administration of both nutritive and LNCS in this current study. Similar findings were reported by Nemoseck et al. (2011) in Sprague Dawley rats administered with honey and sucrose for 33 days. A systematic review on the effect of free sugars on selected cardiovascular risk factors had an unclear effect on

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the blood lipids considering significant heterogeneity among the studies (Fattore et al., 2017). In another systematic review and meta-analysis, artificial and stevia-based sweeteners did not result in any significant increase in total cholesterol (Movahedian et al., 2021b). Nwachuku et al. (2017) also reported similar trend in the total cholesterol in the Wistar rats fed with honey and refined sugar for four weeks.

In the current study, TG concentration was significantly elevated in all doses of stevia, low and medium-dose white sugar administration and medium-dose administration of honey. Stevia levels were generally higher than other sugar groups and therefore had the worst effect on TG. A positive association has been established between plasma concentration of circulating TG and the increased risk in developing coronary heart disease (Sarwar et al., 2010). This outcome from the current study is contrary to that of (Amin & Almuzafar, 2015) where low and high dose saccharine (10 and 500 mg/kg b.wt.) administered to rats for 30 days resulted in reduced TG, TC, HDL and LDL. A meta-analysis of 14 randomized controlled trials of NNS including stevia on lipid profile, suggested that NNS did not have any effect on TG, TC, LDL and HDL. However, further analysis of subgroups revealed that NNS could be related to an increase in LDL which though small was statistically significant (Movahedian et al., 2021b). Alagwu, Okwara, Nneli, & Osim (2011) after 22 weeks of administration of honey to albino rats also reported an increase in TG concentration in the treatment compared to the control group. This emphasizes the role of chronic intake of sweeteners on TG and subsequently cardiovascular risk.

In this current study, long term intake of sweeteners on HDL-C was observed in the group treated with high-dose of white sugar. HDL-C was significantly low in the high-dose white sugar group. HDL-C is known to be protective against heart disease by eliminating “bad” cholesterol, which is a contributory factor in the development of cardiovascular disease, from the blood (Jomard & Osto,

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2020). Increased consumption of sucrose has been linked with metabolic conditions such diabetes, cardiovascular disease, and obesity. One of the plausible mechanisms of sucrose maybe its effect on triglyceride and HDL-C concentration which can facilitate atherogenicity (Johnson et al., 2009; Stanhope, 2016b).

According to epidemiological evidence, increased LDL is a major underlying factor for heart disease and stroke (Silverman et al., 2016). In this current study, LDL cholesterol was lower in all groups compared to the control except for the group on the white sugar medium dose. Whereas the lowest values were in the honey medium dose, stevia low and high-dose and brown sugar high dose groups, differences were statistically significant in comparison with the control. A comparison of the findings of the current study with earlier studies confirms the LDL lowering properties of honey. For instance, in a 14-day study, honey reduced LDL levels significantly (along-side TC and TG) in Wistar rats fed a high fat diet (Nurmasitoh & Pramaningtyas, 2016). In another study, adult male albino rats administered honey (1ml of honey to 10ml of water given once every day) for 22 weeks had significantly lower levels of LDL compared to controls (Alagwu et al., 2011). In a study involving 60 healthy subjects (18–30 years) randomly assigned to two groups in a double-blind trial of honey and sucrose for 6 weeks, the group that received honey demonstrated a decrease in LDL at the end of the study. However, the group that received sucrose, had an increase in LDL (Rasad et al., 2018). In a review of 7 trials involving 370 participants, total cholesterol, LDL and TG were reduced with honey consumption (Alkhalifah et al., 2021b). On the contrary when 60 volunteers with hypercholesterolemia were either given 75 g of sucrose or honey solutions over 14 days, LDL did not change in those receiving honey, but was elevated in those receiving sucrose (Münstedt et al., 2009). Perhaps the duration of the study may have been a

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contributory factor. A systematic review of 23 trials also did not find a significant effect of honey on LDL (Gholami et al., 2022).

In accordance with this present results, previous studies have shown LDL lowering effect with the administration of stevia. A dose-dependent relationship was observed with the administration of aqueous stevia extract (Stevia rebaudiana Bertoni leaves) and low LDL levels in albino rats (Ahmad et al., 2018).The reduction observed in this current study with stevia administration was not dose dependent. It should be noted that, most of the studies examining the effect of stevia on health used the pure aqueous extract whereas in this study a highly processed commercialized stevia product was used. In a 3-arm parallel, triple blind, randomized study on 138 over-weight subjects, a test beverage was spiked with sucralose, sucrose, and stevia. No changes in LDL or oxidized LDL were observed after 60 days (Zafrilla et al., 2021).

Very low-density lipoprotein (VLDL) has been strongly correlated with the development of atherosclerosis as a result of its TG rich content. Very Low-Density Lipoprotein is synthesized in the liver and serves as a vehicle for transporting TG and cholesterol from the liver to the peripheral tissues (Lee, Akhmedov, & Chen, 2022a). Hypertriglyceridemia is associated with the presence of large VLDL particles which may contain 5-20 times more cholesterol than LDL. Its small particle size compared to LDL allows it to quickly penetrate the walls of the artery leading to a sequalae of inflammation of the endothelium, monocyte infiltration, foam cell formation and ultimately, atherosclerosis and CVD (Nakajima et al., 2006; Nakamura et al., 2021).

Furthermore, overproduction of VLDL in the liver is associated with insulin resistance (Sparks et al., 2012). The current study showed an increase in VLDL with the use of both nutritive and LNCS. There was a dose dependent increase in VLDL concentration with brown sugar and stevia

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administration. Contrary to these results, the aqueous extract of Stevia rebaudiana Bertoni leaves is reported to have lowered VLDL from 21.22 ± 5.79 to 19.33 ± 5.95 mg/dL in experimental rats at a dose of 500 ppm for 8 weeks (Ahmad & Ahmad, 2018b). Similarly, (Abo Elnaga et al., 2016) reported remarkable effect of stevia on VLDL in female rats fed for 12 weeks. However, levels of stevia were far above the WHO recommended doses; hence extrapolation of results to humans becomes unrealistic. Also, in this current study a refined stevia sweetener was used as opposed to the pure aqueous extract used in many studies.

5.8 EFFECT OF SWEETENERS ON LIPID RATIOS

Traditionally, in assessing dyslipidaemia as a risk factor for metabolic syndrome and cardiovascular conditions, the individual components of the lipid profile; total cholesterol, TG, LDL, HDL and VLDL are evaluated with emphasis on high LDL and low HDL levels. However, recent studies have highlighted the inadequacy of the individual components in predicting cardiovascular risk especially when they appear normal or moderately high (Olamoyegun et al., 2016). The following lipid ratios: Castelli Risk Index I, Castelli Risk Index II and Atherogenic Index of Plasma were used to predict cardiovascular risk.

The Castelli index I (CRI-I) also referred to as Cardiac Risk Ratio (CRR) is an indication of the build-up of plaques in the coronary arteries (Akpinar et al., 2013). On the other hand, cardiovascular risk is predicted by Castelli Risk Index II. Even though the individual lipid parameters are fairly predictive of CVD, some studies suggest that with lipid values that appear to be normal or somewhat high, the Castelli index I (TC/HDL) and II (LDL/HDL) which examines the relationships between the individual markers is an alternative diagnostic predictor of cardiovascular events (Akpinar et al., 2013; Edwards, Blaha, & Loprinzi, 2017).

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The worst CRI-I was seen in the group on white sugar high dose and the best in the group on honey low dose. CRI-I was also observed to increase with increasing dose of white sugar. Previous systematic reviews have associated the intake of added sugars from sugar-sweetened beverages to an increased risk of cardiovascular diseases (Huang, Huang, Tian, Yang, & Gu, 2014; Narain, Kwok, & Mamas, 2016; Yin et al., 2021). In a recent prospective cohort study by (Kelly et al., 2023), free sugars intake was associated with an increased risk of total CVD and total stroke. In their study, they also statistically modelled the replacement of the free sugars with non-free sugars, which resulted in a reduced risk of developing CVD or stroke. This effect was more pronounced in females than males. In this same study, a 5% increment in the free sugar consumption was associated with an increase in LDL and the reduction in HDL. These results provide further support for the hypothesis that chronic intake of nutritive sweeteners or added sugars poses a risk factor in developing cardiovascular diseases.

Honey has been suggested as a hypolipidemic agent with a positive effect on metabolic health due to the presence of phenolic compounds. These phenolic compounds found in honey have been shown to prevent arteriosclerotic plaques from forming and improving dilation of the coronary artery (Nguyen et al., 2019b). The hypolipidemic effect of honey observed in our studies was reported by (Erejuwa et al., 2016a). In their study, a 3-week administration of honey in alloxan induced diabetic rats resulted in a decrease in CRI-I. A study using albino rats showed significant improvement in the lipid profile and computed CVD predictive index in honey fed rats compared with the control (Alagwu et al., 2011).

For Castelli index II, white sugar high dose had the highest value and stevia high dose had the lowest (0.491 vs 0.231). Castelli Risk Index II, is a strong CVD predictor and strongly correlated with insulin resistance and myocardial infarction (Ama Moor et al., 2017; Salcedo-Cifuentes et al.,

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2019). This finding confirms the association between intake of refined sugars and CVD (C. Huang et al., 2014; Narain et al., 2016; Yin et al., 2021). Both brown sugar and honey low doses had relatively low CRI II compared with white sugar. Polyphenols present in honey are said to be protective of CVD. Polyphenols in honey such as caffeic acid, acacetin, phenethyl esters (CAPE) and quercetin are of great pharmaceutical interest (Nguyen et al., 2019b). Dietary quercetin has been shown to restore endothelial dysfunction by lowering blood pressure in hypertensive rat models (Sánchez et al., 2006). In a previous study, 10 mg/kg of quercetin (avonoid) administered for 5 weeks to Spontaneous Hypertensive Rats (SHR) caused vascular changes and reduced renal and cardiac hypertrophy (Duarte et al., 2001). From the two Castelli Indices, white sugar increases the risk of developing vascular diseases, specifically arteriosclerosis; a major CVD risk factor.

A more reliable, strong and dependable indicator of the risk to cardiometabolic diseases in the general population that is beginning to gain prominence is AIP. Atherogenic Index of Plasma is a product of TG to HDL ratio, both of which are independent risk factors in the development of CVD (Fawwad et al., 2023; Uzunget & Sahin, 2022). Elevated TG is associated with the development of arteriosclerosis whereas a reduction in HDL reduces plasma clearance of atherogenic cholesterol from the blood (Uzunget & Sahin, 2022). It is also strongly associated with the development of T2D, hypertension and metabolic syndrome (Zhu et al., 2018; Li, Kao, Chang, Chen, & Wu, 2021). In a case-control study, AIP was found to be highly and significantly linked with TG, TG, LDL, HDL and CRI-I and II (Cai et al., 2017).

Furthermore, AIP was the strongest parameter associated with coronary artery diseases (CAD) univariately, and an independent factor for CAD multivariately (Zhou et al., 2021). According to the AIP, risk categorisation, there are three levels of risk; low risk (< 0.1), moderate risk (0.1-0.25) and high risk (≥0.25) (Fawwad et al., 2023). Based on the AIP risk classification, with the

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exception of the control group and the group on low-dose brown sugar and low-dose honey, all the other experimental groups were in the high-risk group. Experimental groups on all doses of white sugar and stevia had high risk. The greatest effect on AIP was with stevia administration. A dose dependent increase was observed for stevia with strong statistical differences at all levels compared to the control group. An implication of this finding is the possibility of stevia accelerating the development of arteriosclerosis and increasing the risk of developing insulin resistance and CVD. As a result, stevia may not be a good replacement for nutritive sweeteners. These findings are supported by WHO's recommendation against the use of LNCS (WHO, 2023a). To the best of my knowledge this is the first study to evaluate the effect of commonly consumed sweeteners on AIP.

Furthermore, based on the findings, the control group had the least AIP which may provide support against the intake of free or added sugars. In a recent umbrella review of 73 meta-analyses on 83 health outcomes, dietary sugar consumption has been strongly associated with the cardiometabolic condition; CVD, diabetes, dyslipidaemia and obesity (Huang et al., 2023). Not only is AIP predictive of CAD in T2D but is also an effective factor to consider in preventing CV complications in T2D patients [88]. It is posited that Castelli index I and AIP may characterize reliable markers of residual risk in CAD and suspected patients. Lastly, it may be a very reliable marker in individuals not presenting any of the traditional risk factors for CV disease (Zhou et al., 2021; Mangalesh, Yadav, Dudani, & Mahesh, 2022).

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5.9 EFFECT OF SWEETENERS ON HISTOLOGY OF ORGANS

5.9.1 Effect of Sweeteners on the Histology of the Pancreas

There were no morphological changes in the pancreas of the control rat. However, changes in the pancreas were observed with the groups administered with a high dose of white sugar, a high dose of brown sugar and a medium dose of stevia. For instance, administration of a high dose of white sugar resulted in perivascular infiltration of leucocytes and overstretched pancreatic ducts. Leucocyte accumulation is indicative of inflammation (Editor & Feldweg, 2011). Overstretched pancreatic ducts have also been associated with a high risk of pancreatic cancer (Tanaka et al., 2002).

High dietary sugars have been suggested as a driver in the development of pancreatic cancer in animal models (Dooley et al., 2020). In a previously conducted 7-year prospective study, sugars added to tea and coffee and high sugar foods from soft drinks were associated with an increased risk of developing pancreatic cancer (Larsson et al., 2006). Inflammation of the pancreas, also known as pancreatitis can damage the insulin producing cells of the pancreas thereby leading to diabetes. In a recent review by Ma et al. (2022), high consumption of dietary sugars has been related to the occurrence of low-grade chronic inflammation. Low-grade chronic inflammation is shown to precede the onset of diabetes (Tsalamandris et al., 2019).

Recent epidemiological studies have also confirmed low grade inflammation as an independent risk factor for type 2 diabetes and CVDs (Duncan et al., 2003; Pitsavos et al., 2007; Sharif et al., 2021). Also, in this study, the pancreas of a rat receiving a high dose of brown sugar was characterised with haemorrhage and oedema. Oedema and haemorrhage are both characteristics of an inflamed pancreas (Khedroo, 1957). Among the stevia group, medium-dose administration resulted in mild congestion in the interlobular spaces also typical of inflammation in the pancreas.

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Thus, the histological findings suggest that, high dose white sugar and brown sugar supports the hypothesis that chronic consumption of high doses of sugar increases the risk of CVD and type 2 diabetes. Inflammation of the pancreas, damaging the pancreatic beta cells will result in a diminished insulin secretion and consequently less GLUT4 expression as observed in this study.

5.9.2 Effect of Sweeteners on Skeletal Muscle Fibres

The effect of the sweeteners on the skeletal muscle fibres was more pronounced in rats administered with stevia, causing degenerative changes. Five (5) months of administration of sucrose to older male rats resulted in a decrease in muscle mass (Gatineau et al., 2015). Muscle fibres of low dose stevia had enlargement which could be due to the accumulation of fatty acid in the skeletal muscle. Deposition of free fatty acid in the skeletal muscle can affect the insulin signalling pathway leading to the development of insulin resistance (Park & Seo, 2020).

GLUT4 protein, found in the muscle responds to the insulin signalling. The reduced GLUT4 protein concentration observed in this current study could be attributed to the development of insulin resistance in the muscle. Contrarily, stevioside administered to STZ induced Wistar rats attenuated insulin resistance by enhancing GLUT4 related glucose uptake (Deenadayalan et al., 2021). Variation in the results between these studies can be attributed to the type of animal model, the type of stevia product administered and the duration of the study. In this current study, healthy rats were used as opposed to the diabetic rats used by (Deenadayalan et al., 2021). Also, in their study, a pure stevioside compound whereas a highly refined stevia sweetener was used in this current study. Currently there is limited literature regarding the role of sweeteners on muscle histology which makes the results of this study difficult to interpret in the context of what has already been studied.

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5.9.3 Effect of Sweeteners on the Brain

The main effect of the brain was on the hippocampus. Except for honey, high dosages of all the other sweeteners had a significant effect on the hippocampus often referred to as the learning centre of the brain. It is the part of the brain responsible for learning and memory and it is mostly affected in neuropsychiatric disorders such as Alzheimer's (Anand & Dhikav, 2012). A study was conducted by Villareal, Cruz, Ples, & Vitor (2016) to examine the effect of artificial sweeteners on memory and hippocampal integrity in mice. Aspartame, sucralose, and stevia were administered to the mice for 32 days. At the end of study, all the sweetener groups showed more neuronal damage than the control group. However, mice administered with stevia had the largest amount of neuronal damage in the hippocampus.

Villareal et al. (2016) associated the neuronal death with oxidative stress. According to Ashok & Sheeladevi (2014), chronic intake of sweeteners of artificial sweeteners is associated with an increase in the formation of free radicals which causes oxidative stress and damage to the cells of the brain. These findings are broadly supported by the work of other studies linking the chronic consumption of artificial sweeteners with memory function or hippocampal integrity. In male Sprague Dawley rats, aspartame, sucralose and saccharin were shown to significantly reduce neuronal count at the CA1-CA3 of the hippocampus (Erbaş et al., 2018).

The findings also showed that, a high dose of white sugar resulted in reactive brain cells in the CA3 area of the hippocampus and a high dose of brown sugar showed reactive mononuclear cells outside the hippocampus. Reactive brain cells or reactive gliosis is the brain's reaction to brain injury (Pekny & Pekna, 2016). Hippocampal injury is associated with diseases such as Alzheimer's, affecting the brains ability to form new memories (Shetty, 2014). In an experimental

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study by Shamsi-Goushki et al. (2021b), a high dose of white sugar and a high dose of brown sugar reduced Brain Derived Neurotrophic Factor (BDNF) in male Wistar rats when compared with the control. The reduction in BDNF was more significant in rats on high dose white sugar than rats on high dose brown sugar. Brain Derived Neurotrophic Factor is a neurotrophic factor needed for the growth and survival of the neurons Shamsi-Goushki et al. (2021b). It is mostly located in the following areas of the brain; hippocampus, hypothalamus, brain stem and the spinal cord (Bathina & Das, 2015). Brain Derived Neurotrophic Factor has been implicated in non-communicable diseases such as diabetes. In a systematic review and meta-analysis on BDNF and type 2 diabetes, patients with type 2 diabetes had lower levels of BDNF (Davarpanah et al., 2021).

From the results, honey administration did not result in any significant alteration in the brain of the rats. This may support the hypothesis that honey has a protective effect on brain health. Both animal and human studies have associated the intake of phenolic and flavonoids with brain health. These compounds have been shown to improve the cholinergic system, improves the synthesis of brain derived neurotrophic factor essential for neuronal function, and gets rid of free radicals hence reducing neuronal damage and improving cognition and memory (Zamri, Ghani, Ismail, Zakaria, & Shafin, 2022; Albertini et al., 2023).

5.10 STRENGTHS OF THE STUDY

1. This study employed an experimental approach using an animal model. This allowed the researcher to explore the plausible mechanism underlying the intake of sweeteners and insulin signalling.
2. Sprague Dawley rats were used for this study. These rats are good models for studying conditions such as metabolic syndrome and cardiovascular diseases.
3. An optimum laboratory environment was ensured throughout the study.

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4. To the best of my knowledge this research is the first of its kind to asses in one study the effect of commonly consumed sweeteners on insulin signalling via the GLUT4 pathway and HK 2.
5. Furthermore, this the first of its kind to assess in one study the effect of commonly consumed sweeteners on Atherogenic Index of Plasma.
6. In addition to all the above, this is the first study to examine effect of commonly consumed sweeteners on muscle fibres.

5.11 LIMITATIONS OF THE STUDY

Normal rat cages were used for the study instead of metabolic cages. Individual metabolic cages would have allowed for the researcher to closely monitor each rat's food and water intake and the physical activity of the rats.

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CHAPTER 6

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

6.1 Effect of the sweeteners on energy intake, food efficiency weight and relative organ weight

The study revealed the differential effects of various sweeteners on energy intake, food efficiency ratio, and percent body weight. High dosages of refined nutritive sweeteners (white sugar and brown sugar) and stevia caused an increase in food consumption and the food efficiency ratio. No sweetener administration as observed in the control group was shown to cause the least amount of weight gain whereas consumption of white sugar, brown sugar and stevia significantly impacted weight gain.

6.2 Effect of sweeteners on cardiometabolic risk factors (lipid profile/ lipid ratios, fasting plasma glucose, serum insulin, insulin resistance).

- Excessive intake of white sugar over a prolonged period tended to significantly reduce HDL. Castelli Risk Index I and II were highest with the administration of a high dose of white sugar. Also, CRI-I and II increased dose-dependently with white sugar administration. Also, AIP was highest with the administration of high-dose stevia and increased in a dose-dependent fashion with stevia administration. Taken together, these findings suggest that white sugar and stevia tend to promote the development of arteriosclerosis a risk factor for cardiovascular diseases and metabolic syndrome.
- Intake of sweeteners over time had an impact on the fasting plasma glucose of the rats. In assessing the fasting insulin levels, the control group had adequate levels of fasting insulin whereas fasting insulin decreased drastically with the administration of sweeteners. This was suggestive of beta cell reduction.

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6.3 Effect of the sweeteners on GLUT4 protein concentration

- There was a significant reduction in GLUT4 protein concentration in all sweetener-administered groups compared to the control group. The sweeteners reduced GLUT4 protein concentration which corroborates with the results of the fasting insulin since GLUT4 expression is in response to insulin secretion. Thus, a reduction in insulin secretion will subsequently result in a reduction in GLUT4 concentration. Stevia had the greatest effect on GLUT4 protein concentration.

6.5 Effect of sweeteners on glucose metabolic pathway by hexokinase II

- High doses of the sweeteners resulted in a decrease in HK2 within all the sweetener groups.

6.6 Effect of Sweeteners on the Histology of the Pancreas, Muscles, and Brain

- The main effect of the sweeteners on the brain was on the hippocampus. The representative brain from the control group showed no alteration or changes to the hippocampus. High doses of the sweeteners except for honey caused significant alteration to the hippocampus of the brain.
- The pancreas from the control rat showed a normal islet of Langerhans with pale rounded and ovoid β-cells. However, representative pancreas from the white sugar and brown sugar groups showed perivascular infiltration of leucocytes and hemorrhage indicative of the presence of inflammation which can destroy the pancreatic beta cells. Honey did not cause any significant alteration in the pancreas.

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- A longitudinal section through the muscle fibre of the control rat showed adequately preserved myofibers with clear striation and peripheral myonuclei. The main effect of the sweeteners on the skeletal muscle was observed among the stevia-administered groups.

In summary, chronic administration of the sweeteners significantly impacted the insulin signaling pathway, by significantly altering GLUT 4 protein concentration and Hexokinase II. The chronic administration of sweeteners; particularly white sugar, brown sugar and stevia had negative effects on the cardiometabolic risk factors.

This study fills in the knowledge in examining the effect of same dosages of sweeteners based on the WHO recommendation on cardiometabolic risk factors. Hence providing data on which sweetener is healthier with regards to cardiometabolic health. It also offers valuable insight for the first time on the effect of commonly consumed sweeteners on the insulin signaling pathway via the GLUT4 protein and also on the HK 2. It offers valuable insight on the effect of commonly consumed sweeteners on atherogenicity using the lipid ratios. The study also adds to our understanding on the possible effect of sweeteners on brain health.

6.2 RECOMMENDATIONS

The findings of this study have a number of important implications for future practice;

1. Future studies could explore other effects the sweeteners have on the insulin signalling pathway and the brain.
2. Future studies should consider using both sexes of rats to determine any differences on their metabolic effects with the intake of sweeteners.
3. Future studies should also consider the use of metabolic cages in order to measure the physical activity of the rats.

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4. The study highlighted the negative effects of commercial stevia and white sugar on weight. Hence weight management strategies by dietitians and nutritionists to replace nutritive sweeteners with stevia should not be encouraged. Also cutting back on the intake of white sugar will be a useful approach in weight management.
5. White sugar and stevia have significant effects on atherogenicity. In the dietary management of dyslipidaemia or cardiovascular conditions, cutting back on the addition of refined sugars and commercial stevia sweetener will be useful.
6. Both nutritive and non-nutritive sweeteners had an effect on glucose homeostasis and insulin signaling. This should serve as a guide to dietitians and nutritionists during their public health education on the use of sweeteners in the management or development of diabetes.
7. Honey seemed to have a better effect on cardiometabolic risk factors, hence may be a better replacement to refined sugars when taken in moderation by both healthy individuals and individuals with cardiometabolic conditions. This can be promoted by dietitians and nutritionists during nutrition education.
8. Enforcement of food regulations and policies in addressing the recent upsurge in the use of LNCS in the food industry as a replacement for nutritive sweeteners by the Food and Drugs and Authority.
9. Public health education on the health effects of chronic intake of sugar on health should be promoted by dietitians and nutritionists. Most especially encouraging the consumption fruits rather than the sugar sweetened beverages.
10. More studies on honey which apparently appeared the best must be undertaken to determine the composition of those on the market.

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APPENDICES

Appendix A: Bi-Weekly Measurement of Fasting Plasma Glucose

FBS (mmol/l)	C (n=3)	WSL (n=3)	WSM (n=3)	WSH (n=3)	BSL (n=3)	BSM (n=3)	BSH (n=3)	HL (n=3)	HM (n=3)	HH (n=3)	SL (n=3)	SM (n=3)	SH (n=3)	p-value
Baseline	5.7±0.2	5.8±0.1	6.1±0.2	5.1±0.2	4.9±0.1	5.6±0.2	5.8±0.3	5.5±0.2	4.7±0.1	4.6±0.2	4.4±0.3	4.8±0.3	5.5±0.2	**<0.0001***
Week 3	4.2±0.2	4.4±0.1	3.8±0.2	4.0±0.1	3.8±0.2	4.1±0.2	4.3±0.1	3.9±0.1	3.8±0.02	3.4±0.2	3.4±0.2	3.6±0.1	4.7±0.1	**<0.0001***
Week 5	3.7±0.2	4.3±0.1	4.1±0.2	3.7±0.4	4.1±0.1	4.2±0.2	4.9±0.2	4.1±0.1	4.3±0.2	4.0±0.1	4.6±0.2	4.2±0.1	4.9±0.1	**0.026***
Week 7	4.5±0.6	4.4±0.2	3.9±0.2	4.5±0.1	4.9±0.1	4.5±0.1	4.4±0.1	4.3±0.1	4.0±0.1	3.8±0.2	4.4±0.04	4.2±0.2	4.4±0.2	0.059
Week 9	4.6±0.1	3.8±0.1	4.5±0.1	4.2±0.05	4.6±0.02	4.4±0.5	4.5±0.1	4.9±0.3	4.2±0.2	3.8±0.04	4.2±0.2	3.9±0.2	4.7±0.3	**<0.0001***
Week 11	4.7±0.4	4.5±0.1	4.6±0.1	4.5±0.1	4.6±0.2	4.9±0.02	4.9±0.1	4.5±0.2	4.6±0.1	4.0±0.2	4.7±0.3	4.5±0.1	4.6±0.3	**<0.0001***
Week 13	4.8±0.2	4.4±0.1	4.9±0.2	4.7±0.1	4.6±0.1	4.5±0.1	4.3±0.4	4.20.2	4.7±0.2	4.2±0.2	4.3±0.2	4.5±0.1	4.5±0.1	0.274
Week 15	5.6±0.1	4.8±0.2	4.6±0.05	4.9±0.1	5.1±0.1	4.8±0.2	4.7±0.02	4.8±0.02	4.6±0.1	4.4±0.1	4.6±0.1	4.9±0.1	4.7±0.2	**0.0003***
Week 17	4.7±0.1	4.4±0.1	5.5±0.2	4.8±0.2	5.3±0.1	4.9±0.1	4.8±0.1	4.4±0.2	4.7±0.1	4.1±0.03	4.8±0.03[a]	4.4±0.2	4.7±0.1	**<0.0001***

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Appendix B: Ethical Clearance Letter

UNIVERSITY OF GHANA

University of Ghana Institutional Animal Care and Use Committee
(UG-IACUC)

Phone:
Email: UG-IACUC@ug.edu.gh

P.O. Box LG 581
Legon, Accra
Ghana

Office Location: Department of Animal Experimentation Building, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana

11/04/2022

ETHICAL CLEARANCE
(UG-IACUC 001/21-22)

Your protocol for an ethical clearance has been reviewed by the University of Ghana Institutional Animal Care and Use Committee and has been approved as follows:

TITLE OF PROTOCOL: Action of nutritive and non-nutritive sweeteners on insulin signaling pathway and cardiometabolic risk factors using experimental animal models

PRINCIPAL INVESTIGATOR: Ruth Tenkoramaa Owu

Please note that the final review report must be submitted to the Committee at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to UG-IACUC for review and approval prior to implementation.

Please report all serious adverse events related to this study to UG-IACUC within seven days verbally and in writing within fourteen days.

This certificate is valid till 10st April, 2023. You are to submit annual reports for continuing review.

Signature of Chairperson
Prof. Major (Rtd.) George A. Asare

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