THE EFFECT OF A SUGAR SWEETENED BEVERAGE DIET ON DNA METHYLATION IN A CACO-2 CELL LINE IN VITRO

BY

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DISSERTATION SUBMITTED FOR THE FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MAGISTER TECHNOLOGIAE IN BIOTECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

FACULTY OF APPLIED AND COMPUTER SCIENCES

VAAL UNIVERSITY OF TECHNOLOGY

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DECEMBER 2020

DECLARATION

The experimental work illustrated in this dissertation was conducted in the Biotechnology research laboratories, Faculty of Applied and Computer Sciences, Vaal University of Technology, under the supervision of Dr.C.C Ssemakalu and co-supervision of Prof. M. Pillay.

The results reported in this document were obtained experimentally with reference from the work of other researchers acknowledged throughout the thesis. I, ***NDHLOVU L***, declare the above statement as authentic.

Ndhlovu L.

DATE: 13 DECMBER 2020

ACKNOWLEDGEMENTS

I would like to return all praise and gratitude to the King of glory, the Creator, for His unending mercies day after day. He always renewed my strength through the hardships I went through.

This research might not have been possible if it was not for the continued support, constructive criticism and love shown by my supervisor, Dr. C.C Ssemakalu. His works speak volumes, and the wisdom he has imparted to me is second to none.

Forgoing out of his way and making sure that he edited my research, I would sincerely love to thank my co-supervisor, Prof. M. Pillay.

Gratitude also goes to Dr. S. Takaidza and Mr. G. Mohlala, for their continued support and mentoring. I would also love to thank the Vaal University of Technology for allowing me to use their laboratories to conduct the research and custodians from labs F305 and F309 for always making sure that I did my work in the mostconducive environment.

I want to pass my heartfelt gratitude to my fiancé Mr. Thabang Mkhonto for his undying support throughout the years, for his love, wisdom and patience. I am forever indebted to him for not giving up on me and in believing in my work.

The National Research Foundation has my utmost gratitude for covering my tuition fees (Grant number: AEMD170712252564).

To my family, especially my parents (Michael and Margareth Ndhlovu) who have loved and raised me to believe in my dreams and abilities, I have no words to thank you for the continued support I have received from you. I thank God daily for your lives, and I pray God abundantly blesses you.

DEDICATION

I dedicate this work to Keneilwe, Vutlhari Ndhlovu, Lisakhanya and Zanengcebo Mkhonto. May they grow up to witness and understand the works of my hands, and the blessing of wisdom the Almighty endowed me with.

ABSTRACT

Obesity has steadily increased and represents a major public health problem worldwide, reducing quality of life and causing a range of health problems. Obesity has emerged as the fifth leading risk of global deaths. Annually, 2.8 million adults die as a result of being overweight or obese. The increase of obesity remains inexplicable in terms of genetic susceptibility to obesity. The genetic loci identified by genome-wide association studies (GWASs) explains about 2% of the heritability for obesity. Perhaps other factors such as epigenetics may be involved in the increase of obesity and may offer solutions for the management of obesity. Epigenetics is defined as a heritable change in gene expression without altering the genome sequences. It may help in providing a logical explanation between the genome and environment which shapes obesity risk and may help to explain the "missing heritability". Epigenetics may affect two mechanisms, namely: i) DNA methylation,and ii) histone modifications. DNA methylation might give scientists a link to the rise in obesity.The study aimed to investigate the effect of sugars used as sweeteners in sugar-sweetened beverages (SSB) on DNA methylation in a Caco-2 cell line *in vitro*. Four major objectives were pursued in the study which were to:(1) stimulate the Caco-2 cells with varying concentrations of sugar sweeteners and assess the morphological changes of the cells; (2) evaluate the cytotoxicity of different concentrations of the sugar sweetener on the Caco-2 cell line using the Alamar blue and LDH assay; (3) obtain genomic DNA from the treated Caco-2 cell line and perform bisulfite conversion and rest; and (4) amplify the WT1, MEG3, TNFRSF9, ATP10A, and CD44 obesity-associated genes and ascertain their degree of methylation.

Caco-2 cells were stimulated with sugar sweeteners at varying concentrations (low, medium and high) for an incubation period of 62 days,and images of the cells were captured for morphological characterisation. The incubation condition entailed cells plated in a 12 or 96 well plate, incubated in a humidified 5% CO_2 incubator at 37 °C and there is nutrient renewal every three days.Alamar blue, a cell proliferation colourimetric assay and lactate dehydrogenase assays (LDH), a homogenous membrane fluorimetric assay were used for the cytotoxicity studies. The results of the characterisation showed that different concentrations of sugar sweeteners affected the morphology of the cells as the incubation period progressed. The cytotoxicity results

of both LDH and Alamar blue depicted low concentration of sweeteners that had low-to-moderate toxicity and the medium and high concentration of the sweeteners had a moderate to high toxicity on the Caco-2 cells. DNA from the Caco-2 cells was extracted. Techniques used to study DNA methylation such as bisulfite conversion, PCR amplification and restriction enzymes that have differential sensitivity to 5-methyl-cytosine were performed. The quality of DNA extracted was good. The bisulfite conversion was conducted andno amplification was observed, as a contingency plan Normal PCR was performed to amplify the CpG islands, and there was amplification.

In conclusion, the study showed that a low concentration of a sugar sweetener (fructose: glucose) used in beverages had low toxicity to the Caco-2 cell line and prolonged exposure of the low concentration might have an adverse effect on the cells' morphology. At medium concentrations, the sugar sweetener used in beverages had medium toxicity to Caco-2 cells; prolonged exposure may lead to morphological changes. These findings indicated that control of dietary glucose intake is an important strategy in combating the development of obesity and type-2 diabetes**.** DNA methylation could not be established.

Keywords: Caco-2 cell line, Alamar blue assay, LDH assay, DNA Methylation, Epigenetics, Obesity, Sugar-sweetened beverages, Sugar sweeteners

ABBREVIATIONS

ANOVA	Analysis of variance
ATP10A	ATPase10
BMI	Body mass index
Caco-2	Cancer Coli-2
CD44	Cluster of differentiation 44
CKK	Cholecystokinin
CpG	Cytosine phosphate Guanine
CNS	Central nervous system
CVD	Cardiovascular disease
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferases
FBS	Foetal bovine serum
GH	Growth Hormone
GLP	Glucagon-like peptide
HFCS	High fructose corn syrup
IGF-1	Insulin-like growth factor- 1
MEG 3	Maternally Expressed 3
MiRNA	Micro Ribonucleic acid
OXM	Oxyntomodulin
PCR	Polymerase Chain Reaction
SSB	Sugar-Sweetened Beverage
T2D	Type-2diabetes
TF	Transcription factor

TNF	Tumour Necrosis Factor
TNFRSF9	Tumour necrosis factor receptor superfamily 9
WHO	World Health Organisation
WT1	Wilm's Tumour-1

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CHAPTER ONE

BACKGROUND

1.1. Introduction

The number of overweight people in the world is estimated to be over two billion of which approximately 25% are obese (WHO, 2014). The prevalence of obesity has steadily grown from being an epidemic to a pandemic. Obesity occurs as a result of an abnormal accumulation of triglycerides in the adipocyte tissues. The etiological agents of obesity are ambiguous and may include compounds that interact with the neuroendocrine system, environmental factors, stress, unhealthy dietary habits, lack of exercise and genetic variation (Milagro *et al.,* 2011).

Drinking excessive sugar-sweetened beverages (SSBs) may also lead to obesity. The term sugar-sweetened beverages has no official definition. However, as a convention, SSB can be defined as beverages with added sugar. Although SSB are a source of calories, the added sugar contains excessive calories contributing to weight gain and obesity. The most common sugars that are used are sucrose, fructose, and glucose. Evidence suggests that the consumption of sugar-sweetened beverages (SSB) has increased in parallel to overweight and obesity trends (Hu, 2011). Currently, SSB contribute between 10% and 15% of the caloric intake of young people between the ages of 15 to 24 (Hu, 2011).

In the quest for obtaining solutions for the management of obesity, more than 40 genes have been linked to obesity and fat distribution (Milagro *et al.,*2011). However, these genetic variants do not fully explain the heritability of obesity. Despite advances in understanding the genetic variants, lifestyle factors and gene-environment interactions associated with obesity (Berndt *et al.,* 2013), much of the inter-individual variation in body weight remains unexplained by measurable lifestyle and genetic factors (Mendelson *et al.,*2017). As a result, epigenetics has been considered (Herrera *et al.,*2011) to understand gene expression as an etiologic agent of obesity. Epigenetics is the study of heritable changes in gene expression that occur without altering the DNA sequence (Bird,2007). Epigenetic effects in humans may be stable over time but can change due to different factors, most of which are environmental and dietary. Research attributes the occurrence of epigenetic effects on mechanisms such as DNA methylation and covalent histone modifications (Herrera *et al.,*2011). Regulatory RNAs such as microRNAs (miRNAs) and large non-coding RNAs (lncRNAs) have also been shown to contribute to the occurrence of epigenetic effects (Milagro *et al..* 2011).

Mechanisms resulting in epigenetic effects are often induced by a combination of environmental and dietary factors such as methyl donors, polyphenols, and some minerals (Milagro *et al.,* 2011).

DNA methylation entails the covalent transfer of a methyl group to the C-5 position of the cytosine ring of DNA by DNA methyltransferases (DNMTs) (Robertson, 2005). DNA methylation often occurs in CpG islands (Herrera *et al.,* 2011) which are regions of DNA where C (cytosine) occurs next to G (Guanine) in a linear sequence. When the promoter region contains a CpG island that is methylated, the expression of the gene is repressed (Razin and Cedar, 1991; Bird, 2002). DNA methylation plays a critical role in processes including genomic imprinting, X-chromosome inactivation, suppression of repetitive element transcription, gene silencing, and transposition (Robertson, 2005; Gopalakrishnan *et al.,*2008; Jin *et al.,*2008).

Methylated DNA influences gene expression through its interaction with both chromatin proteins and specific transcription factors (Razin and Cedar, 1991). DNA methylation, the most frequent and well-characterised epigenetic modification, reflects at the molecular level. DNA methylation is influenced by environmental exposure and genetic factors(Relton *et al.,* 2010). DNA methylation has the potential to affect an individual's susceptibility to obesity (Van Dijk *et al.,* 2014). Furthermore, changes in the methylation of DNA may occur secondarily to obesity (Mendelson *et al.,*2017).

Common cell lines used for obesity studies include X3, X9 3T3-L1 preadipocyte and Caco-2. In this study, the Caco-2 cell line was used as a model to investigate the link between sugar sweeteners used in beverages and DNA methylation. This cell line originates from the parental cell line of human colon adenocarcinoma. The Caco-2 cell line undergoes a culture of spontaneous differentiation leading to the formation of a monolayer of cells, expressing several morphological and functional characteristics of a mature enterocyte (Sambuy *et al.,* 2005). The Caco-2 cell line was chosen as a model organism over 3T3-L1 preadipocyte because Caco-2 cells can retain a high proliferation potential resulting in a cell population. The characteristics and functionality of the Caco-2 cell line makesit an ideal choice when investigating related dietary conditions such as obesity.

In this study, the potential for sugar sweeteners used in beverages to induce epigenetic alternations such as DNA methylation was investigated. The CpG islands

of five obesity-related genes namely Wilm's Tumour (*WT1*), Cluster of differentiation(*CD44* molecule c), Tumor Necrosis Factor Receptor Super Family member 9 (*TNFRSF9*), ATPase class V type 10A (ATP10A) and Maternally Expressed 3 (*MEG3*) (Milagro *et al.,*2011) were assessed for DNA methylation following the stimulation of Caco-2 cells with sugar sweeteners used in SSB diet.

The limitation of this study is that the Caco-2 cells do not display any phenotypical change and only genetic analyses were performed. The Caco-2 cell line is ideal for preliminary studies, but *in vivo* work provides a better idea of how sugar sweeteners may influence obesity.

1.2. Motivation

More than 1.9 billion people who are 18 years and older are overweight, globally. Of these, over 600 million are obese. Projections indicate that this figure will rise to 2.16 billion overweight and 1.12 billion obese individuals by 2030 (WHO, 2014). In South Africa, the prevalence of obesity among adults sharply increased during the 1990s and early 2000s (WHO, 2003). The proportion of the South African population categorised as obese (BMI 30 kg/m^2 or over) increased from 13.2% in men in 1993 to 24.3% in 2014, and from 16.4% in women in 1993 to 26.8% in 2014 (Cois and Day, 2015). By 2050 the prevalence of obesity in South African adult men and women is predicted to reach 60% and 50%, respectively (Cois and Day, 2015). Current attempts to reduce obesity through proper diet, exercise, and education, surgery, and drug therapies are failing to provide effective long-term solutions to this pandemic. The current research paradigm on obesity suggests that this condition may be linked to the genetics of the individuals.Furthermore, more than 40 genes have been linked to obesity and fat distribution (Milagro *et al.,*2011). However, the variations in these genes do not fully explain the heritability of obesity. Therefore, variation resulting from epigenetic events should be considered.

1.3. Problem statement

The consumption of SSB has been associated with the emergence of obesity among adults and children (Wilga *et al.,*2010). Obese children are more likely to fall ill, abstain from school, experience health-related limitations, and require extra medical attention

than normal-weight children (Wilga *et al.,*2010). Obese children are also susceptible to ailments such as asthma, Type-2 Diabetes, and cardiovascular diseases. In addition, obese children tend to suffer from emotional trauma and low self-esteem. In adults, obesity has been associated with a large number of health conditions, including heart disease, stroke, diabetes, high blood pressure, unhealthy cholesterol, asthma, sleep apnea, gallstones, kidney stones, infertility, and as many as 11 types of cancers, including leukaemia, breast, and colon cancer (School of Public Health, 2016). A proper understanding of the etiological agents of obesity is required. Current approaches towards understanding the aetiology of obesity need to consider the role played by SSB in obesity.

1.4. Research aim

To investigate the effect of sugar-sweetened beverages (SSB) on DNA methylation in a Caco-2 cell line *in vitro.*

1.5. Research objectives

The specific research objectives were:

1. To culture of the Caco-2 cell line *in vitro.*
2. To stimulate the Caco-2 cells with varying concentrations of sugar sweeteners and to assess the morphological changes of the cells.
3. To evaluate the cytotoxicity of different concentrations of the sugar sweeteners on the Caco-2 cells using the Alamar blue and LDH assays.
4. To obtain genomic DNA from the treated Caco-2 cells and carry out bisulfite conversion.
5. To amplify the *WT1*, *MEG3*, *TNFRSF9*, *ATP10A*, and *CD44* obesity genes and ascertain their degree of methylation.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiology of obesity

Obesity can be defined as a health-threatening disorder that results from excess fat accumulation in the adipose tissue (WHO, 2003). Obesity at a physiological level occurs when there is excessive storage of abnormal amounts of triglycerides in adipose tissue. These triglycerides are then released from adipose tissues as free fatty acids with detrimental effects (O'Rahilly, 2009).The excessive fat storage results in an imbalance characterised by a higher than average energy intake and reduced energy expenditure (Herrera *et al.,* 2011). This continuous imbalance then leads to weight gain. According to Herrera *et al.,* (2011), obesity is a pandemic that directly and/or indirectly affects and threatens public health. Obesity affects people's lives negatively and imposes excessive financial implications on health systems(Maria and Evagelia, 2009). It is a severe pathologic situation that causes both morphological and functional disorders in the human body and is associated with a high risk of morbidity and mortality (Maria and Evagelia, 2009). With the high prevalence of this pandemic, the scientific community is obliged to understand and find solutions for obesity through research.

According to the WHO (2003), obesity is classified as a chronic and severe disease in developed and developing countries affecting both adults and children (WHO, 2003). Furthermore, obesity has tripled in developing countries especially in low-income countries in the past twenty years (Borg *et al.,* 2005). In 2016, a study conducted by the World Health Organisation revealed that more than 1.9 billion adults (18 years and older) were overweight (WHO, 2017). Of these, over 650 million adults were obese, 39% of adults (39% of men and 40% of women) were overweight (WHO, 2017). Overall, about 13% of the world's adult population (11% of men and 15% of women) were obese in 2016, and the worldwide prevalence of obesity nearly tripled between 1975 and 2016 (WHO, 2017). An increase in the Body Mass Index (BMI) to between 30 and 35 has been associated with the deaths of more than 2.5 million people annually and this figure is expected to double by 2030 (Andreyeva *et al.,* 2007; Torjesen, 2007; Berghofer *et al.,*2008).

The distribution of obesity varies among different geographical regions (Figure 2.1). In Southern Africa, particularly South Africa, an unusually high increase in BMI has been observed (Finucane *et al.,* 2011; Cois and Day, 2015). Between 1998 and 2008, the

estimated proportion of the South African adult population that was considered overweight or obese increased from 29.1 to 31.1% among males (+6.9 % in relative terms), and from 56.2 to 59.5 % among women (+5.9 %) (Ardington *et al.,* 2009). Between 2016 and 2018, South Africa has been declared as the country with the highest obesity rate in the sub-Saharan region (Heala.org, 2018).

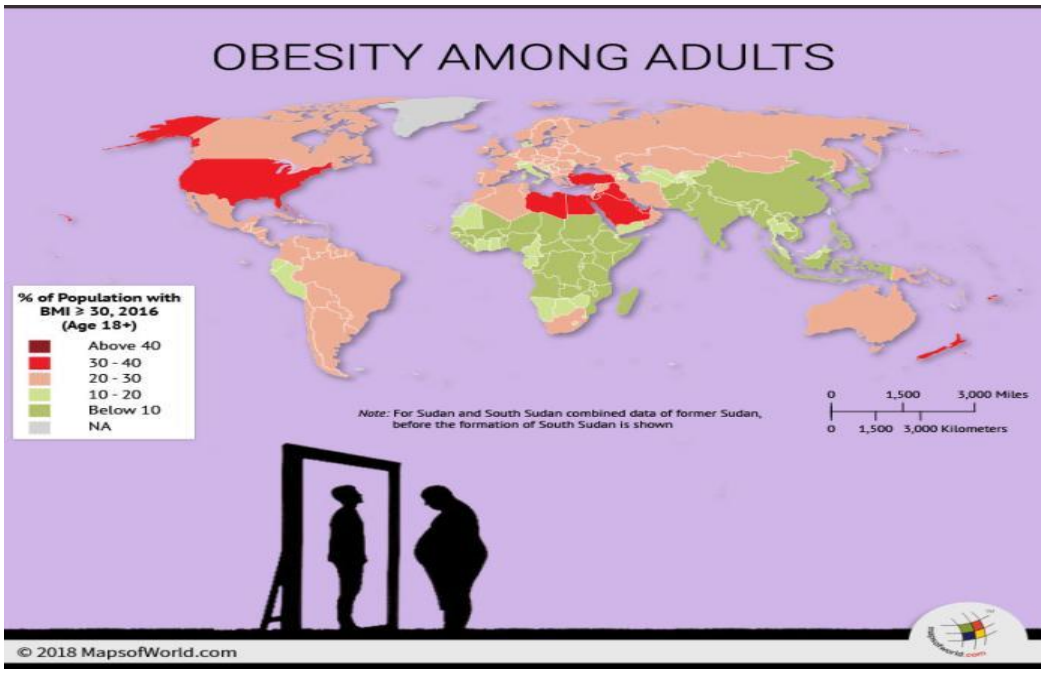

Figure 0.1: The distribution of obesity globally (Adapted from Maps of World 2018, www.mapsofworld.com)

2.2 Assessment of obesity

There are two types of body fat namely, essential and storage fat. Essential fat is necessary for the normal functioning of the body and is mainly stored in the bone marrow, heart, lung, liver, spleen and muscles (Maria and Evagelia, 2009). Essential fat also includes the female fat, which is stored in the breasts and hips and plays an essential role in child-bearing (Kravitz and Heyward 1992). Storage fat is located around internal organs and directly beneath the skin. Storage fat provides bodily protection and serves as an insulator to conserve body heat (Kravitz and Heyward, 1992). In healthy young adults, the total body fat constitutes about 15-20% of total body weight for men and 20-25% for women (Polikandrioti *et al.,*2009). However, it should be noted that fat distribution differs according to where it is found (either central

or regional) (Maria and Evagelia, 2009).

Fat in the central area results in central obesity, which is characterised by the accumulation of fat in the upper torso, specifically the abdomen. Central obesity is shared among the male population (Recio-Rodriguez *et al.,*2014). Excessive fat in the regional area results in regional obesity which is characterised by the deposition of fat in the thighs and hips. Regional obesity is common in the female population (Recio Rodriguez *et al.,*2014).

Accurate measurement of the amount of fat requires the use of methods and equipment that is often exclusive to medical and research laboratories.In clinical practice, more simple techniques such as the weight - height tables, the Body Mass Index (BMI) assessment, and the skin fold measurement are used. The BMI is a quantification of the amount of muscle, fat, and bone in an individual and then categorises that person asunderweight, average weight, overweight or obese (Kendrick, 2015). Tables are often used to indicate an acceptable weight range for height and gender. Therefore, these tables could be used to classify an individual as underweight, healthy or overweight (Maria and Evagelia, 2009). A BMI greater than 30 is an indication that the individual is obese. Commonly accepted interpretations of BMI ranges are shown in Figure 2.2 following.

Figure 0.2: Different levels of body mass index (BMI) (Adapted from, www.everydayhealth.com)

The skinfold measurement technique is the simplest method for measuring body fat percentage and the results are obtained from specific tables.Waist Circumference (WC) provides essential information about the accumulation and distribution of body fat (Maria and Evagelia, 2009).More specifically, it is considered an adequate tool for assessing central obesity. In addition, the ratio of Waist to Hip (WHR) is another secure method for assessing central obesity. WHR is defined as the ratio between the lower part of the crest of the iliac ala and the perimeter of the hips measured at the level of trochanters (Kushner and Roth, 2003).

2.3 Consequences associated with obesity

There has been a rise in the prevalence of obesity over the last 30 years. This surge in obesity has been linked to urbanisation and modernisation (Dixon, 2010). More than 300 million people worldwide now exceed the obesity BMI threshold of 30 kg/m^2(Kelly *et al.,* 2008). On average, those with obesity may have their life span shortened by six to seven years (Dixon, 2010). Obesity is associated with a wide range of health issues ranging from specific diseases such as type-2 diabetes and hypertension to impaired quality of life, psychosocial disturbance, and limited access to quality care (*ibid.*).

2.3.1 Mortality

It is known that obesity can kill a person. With the current trend, at least 2.8 million people die each year as a result of being overweight or obese(WHO, 2017). Mortality risk increases with an increasing BMI in the range above 30 (Gu *et al.,* 2006; Jee *et al.,* 2006). The actual risk of death associated with obesity increases with age and BMI (Byersa, 2006), but the estimated years of life lost as a result of obesity are greatest in younger adults (Fontaine *et al.,* 2003). The causes of increased mortality related to overweight and obesity include cardiovascular diseases, diabetes, and kidney diseases, and obesity-related cancer deaths (Flegal *et al.,* 2007).

2.3.2 Quality of life

Obesity has a significant impact on a patient's physical, mental, psychosocial, and economic health (Dixon, 2010). These conditions have an impact on the patient’s health-related quality of life and arean important reason for seeking medical interventions(Doll *et al.,* 2000; Katz *et al.,* 2000; Dixon *et al.,* 2001). Estimates suggest

that obesity has a more significant negative impact on quality of life than 20 years of natural ageing (Sturm, 2002a).

2.3.3 Economic Burden

Obesity imposes a significant burden on the economy at both micro-and macro-levels(Some *et al.,* 2014). At a micro-level, obesity imposes a substantial burden on the individual. According to McCormick (2006), morbidity, mortality, social exclusion, discrimination, sickness, and under-productivity increase with levels of obesity. At a macro-level, pressure on the healthcare system, a reduction in the national output level, a reduction in tax revenue, increased government expenditure on incapacity and unemployment benefits, and increased operating costs for businesses are all affected with increased levels of obesity (McCornick, 2006). There appears to be a negative relationship between obesity and employment in the labour market(Lindeboom *et al.,* 2003; Some *et al.,* 2014).

2.3.4 Psychological effect

There is evidence which shows that obese people face stigmatisation, job discrimination, social exclusion, and less satisfactorytreatment in educational and healthcare settings (Myers and Rosen, 1999; Puhl and Brownell, 2001). Obese people face “body shaming” and are subjected to bullying and teasing, that often resultin depression and low self-esteem.

2.3.5 Diabetes

Diabetes mellitus (DM) is a chronic disorder in which carbohydrate, protein, and fat metabolism are altered. The disorder is caused by the absence of insulin secretion due to the inability of the β-Langerhans islet cells of the pancreas to produce insulin or to defects in insulin uptake in the peripheral tissue (Al-Gobhan *et al.,* 2014). DM is broadly classified into two categories, which includes type 1 and type 2 diabetes (Scheen, 2003). According to the WHO (2017), 171 million people worldwide are diabeticand this number would increase to 366 million by 2030. Approximately 6% of the South African population is diabetic (Pheiffer *et al.,* 2018). The risk of diabetes increases with the severity and duration of obesity and with a more central distribution of body fat (Bray, 2004).

The primary factor in the pathophysiology of type 1 diabetes is considered to be autoimmunity (Mathieu and Badenhoop, 2005). Type 1 diabetes has not been linked to obesity, whereas type 2 has been closely correlated with obesity (Dixon, 2010). Obesity and type-2 diabetes (T2D) are likely to be the two greatest public health problems in the future (Zimmet *et al.,* 2001; Dixon, 2010) since they are associated with insulin resistance (Al-Gobhan *et al.,* 2014). Although body fat distribution is an important determinant of increased risk of diabetes, the precise mechanism of association is not clear (Eckel *et al.,* 2011; Neeland *et al.,* 2012).

2.3.6 Cancer

Obesity may contribute towards the causes of cancer through several mechanisms. There is evidence that links insulin resistance, which often accompanies obesity, with increased cancer risk (Bordeaux *et al.,* 2006). Insulin influences cell growth and inflammation in several ways and may promote cancer. One potential mechanism involves promoting the production of insulin-like growth factor 1 (IGF-1), which stimulates cell proliferation and inhibits cell death in laboratory studies (Bordeaux *et al.,* 2006). Increased cell proliferation and decreased cell death can promote the development of cancer. Several studies have shown that obese people have higher-than-usual levels of the biologically active form of IGF-1 in their blood and are thus at increased risk of cancer (Frystyk *et al.,* 1995; Nam *et al.,* 1997).

2.3.7 Cardiovascular Diseases

Cardiovascular disease (CVD) is one of the most serious non-communicable diseases in the world. Obesity impacts cardiovascular function and increases the risk of cardiac failure (Dixon, 2010). Increased obesity-related cardiac output contributes to the eccentric and concentric hypertrophy characteristic of obesity-related cardiac failure. Obesity cardiomyopathy is independent of the cardiac effects of hypertension, diabetes, and atherosclerosis (Wong and Marwick, 2007). One reason for the increasedrisk of heart failure is that the heart has to work harder to circulate blood to a larger body (Bray, 2004). The increased cardiac work associated with being overweight may promote heart failure even in the absence of hypertension or other established risk factors for this condition (Bray, 2004).

2.4 Causes of obesity

The exact causes of obesity remain unclear (Apovian, 2016). There appears to be a complex relationship between biological, psychosocial, and behavioural factors, which include genetic makeup, socioeconomic status, and cultural influences (Skelton *et al.,* 2011). Obesity has been associated with gut microorganisms, epigenetic changes, increasing maternal age, lack of sleep, endocrine disruptors, and intrauterine and intergenerational effects (McAllister *et al.,* 2009). A list of the causes of obesity can be found in Figure 2.3.

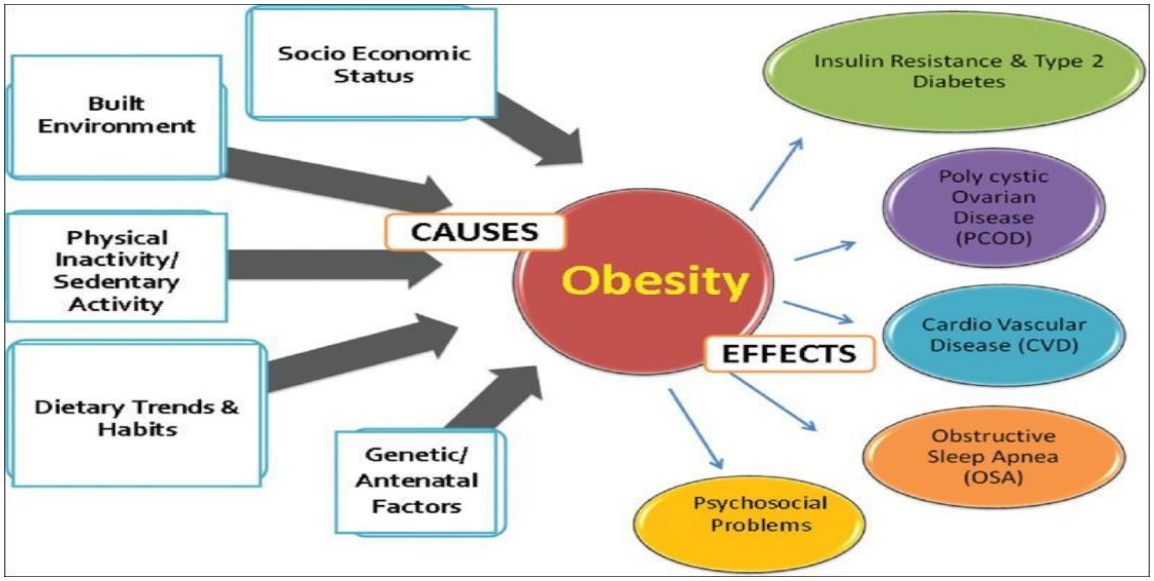

Figure 0.3: Causes and effects of obesity (Adapted from https://www.123rfphotos.com)

2.4.1 Gut microbiome

The body's microbiome consists of different bacteria, viruses, archaea, and eukaryotic microorganisms. These microorganisms have the potential to impact the physiology of the human body in several ways, including contributing to metabolic functions(Schreiner *et al.,* 2015). Studies have demonstrated that the gut microbiome can increase dietary energy harvest, and an "obese microbiome" results in higher total body fat than a "lean microbiome" (Turnbaugh *et al.,* 2006).

2.4.2 Food choices

Food choices are influenced mainly by the home, childcare, school, workplace, and community environments. This directly affects the type and amount of caloric intake (Apovian, 2016). Over the last 100 years, because of technological advances in food processing, the types of foods consumed have evolved. Readily available foods in this era are low in fibre and are unusually high in fat, simple sugar, salt, and they are typically cheaper than healthier alternatives (*ibid.*).Consequently, the excessive consumption of these readily available foods has led to a calorie increase in consumers (Crino *et al.* 2015). An increase in daily calorie intake has been associated with an increase in waist circumference and BMI (Savage *et al.,* 2008; Du *et al.,* 2009). The lack of physical exercise prevents the body from burning off the extra energy. As a result, the extra energy consumed is stored by the body as fat for later utilisation(Gordon-Larsen *et al.* 2006).

2.4.3 Sugar-sweetened beverage diet

There is no clear definition of sugar-sweetened beverages. However, SSB are drinks to which sugar is added as a sweetener. Sugar-sweetened beverages include soft drinks/sodas, flavoured juice drinks, sports drinks, sweetened tea, coffee drinks, energy drinks, and electrolyte replacement drinks, as shown in Figure 1.4(Harrington, 2008; Bleich *et al.,* 2009; Lin and Smith, 2010).The SSB contain added sugars such as sucrose or high fructose corn syrup. For instance, a 330 ml portion of sugar-sweetened carbonated soft drink typically contains almost nine teaspoons of sugar, which translates to approximately 140 kcal of energy, with little to no nutritional value (National Department of Treasury, 2016). The consumption of SSB increased by 135% between 1977 and 2001 (Nielsen & Popkin, 2004). Currently, SSB contribute between 10% and 15% of youth's caloric intake and are the primary source of added sugar in the diet of children and adolescents (Hu and Malik, 2010).Since 1998, the market for soft drinks in South Africa has more than doubled from 2,294 billion litres to 4,746 billion litres in 2012 (Réquillart and Bonnet, 2015). The consumption of SSB is associated with an increase in the risk of obesity at a rate of 1.6 times per additional serving of sugar-sweetened drink consumed daily (Ludwig *et al.,*2001).

The WHO (2013) has expressed concerns that the increased intake of free sugars, particularly in the form of SSB, may increase overall energy intake. This may reduce the intake of foods containing more nutritionally adequate calories, leading to an unhealthy diet, weight gain, and increased risk of non-communicable diseases. The WHO's global action plan encourages member states to consider the implementation of sugar taxes and subsidies (WHO, 2013).

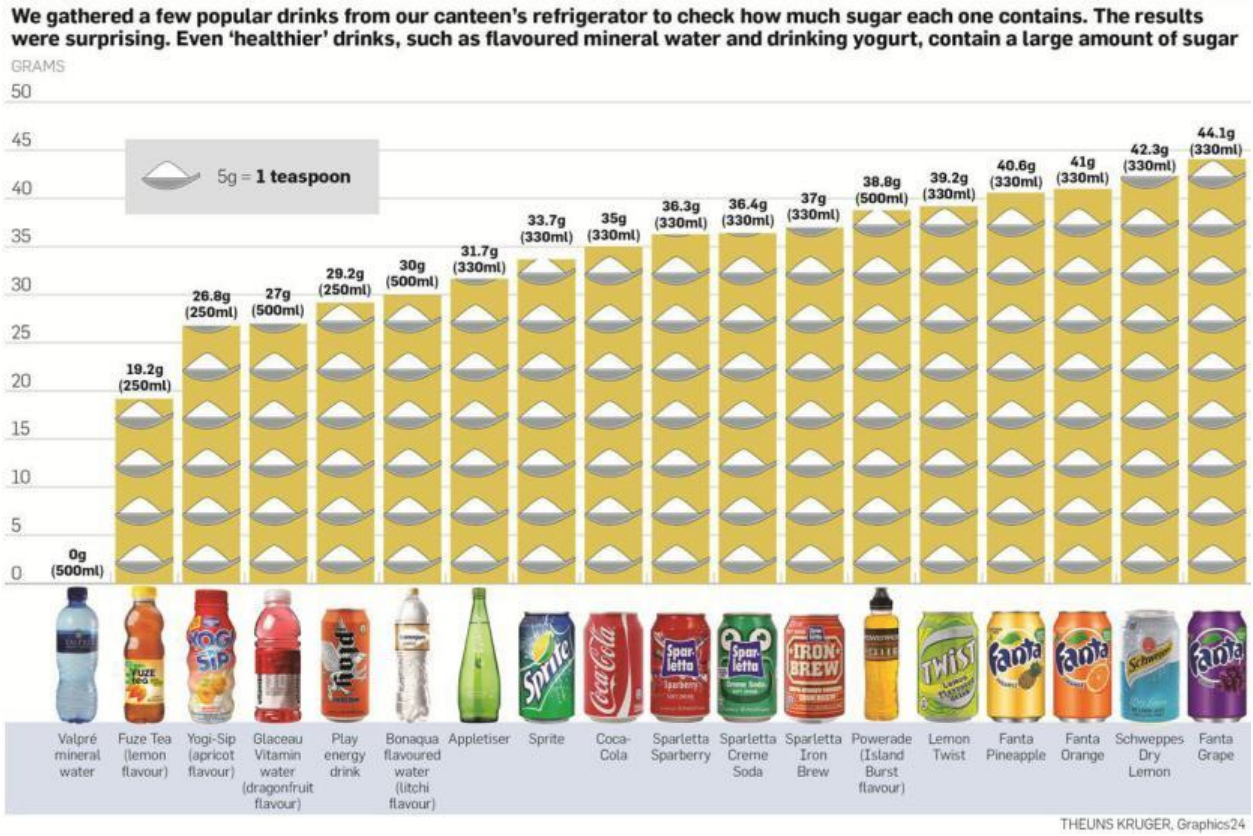

Figure 0.4 Different types of sugar-sweetened beverages and their sugar content (Source: Carteblanche.com, 2018)

2.4.3.1 Composition of the sugar-sweetened beverages

The most common sugars added to fizzy drinks are fructose and glucose in different concentrations(Walker *et al.,* 2014). These sugars are derived from corn starch such as high fructose corn syrup 55 (HFCS 55; 55% fructose-41% glucose), HFCS 42 (42% fructose-52% glucose), and corn syrup (glucose and oligo-glucose with trace amounts of fructose) (Sun and Empie,2012). Glucose is the preferred form of circulating sugar in animals. The body processes most carbohydrates that are eaten as glucose to be used up immediately or stored up in cells. Fructose is abundant in fruits and vegetables. Unlike glucose, the metabolism of fructose is quicker than that of glucose

(*ibid.*). Fructose is not a preferred source of energy for the brain and muscles *(Li et al.,* 2007) because its metabolism has been documented to have a negative effect on the regulation of energy intake and expenditure (*ibid.*).

2.4.4 Endocrine dysregulation

The regulation of food intake is managed by neural and hormonal signals between the digestive and central nervous systems (CNS). Hormones, such as glucagon-like peptide (GLP), oxyntomodulin (OXM), leptin and cholecystokinin (CCK), signal to essential areas in the CNS involved in appetite control (Simpson *et al.,* 2012; Bataille and Dalle, 2014). Blood concentrations of these hormones are often proportional to the required caloric intake (Simpson *et al.,* 2012). Nevertheless, a hormonal imbalance can result in weight gain.

2.4.5 Growth hormone deficiency

Growth hormone (GH) deficiency is the most common hormonal deficit observed in pituitary disease usually resulting in hypopituitarism (Karam and McFarlane, 2007). Growth hormone plays significant roles in regulating energy expenditure, body composition, bone mineral density, lipid metabolism, and cardiovascular function. Notably, GH inhibits lipoprotein lipase, increases hormone-sensitive lipase, and stimulates adipocyte lipolysis (Dietz and Schwartz, 1991). GH enhances protein synthesis and increases muscle and bone mass. GH deficiency in adults is associated with decreased muscle mass, increased truncal fat deposition and reduction of total body water, with a net weight increase of 3.6–7.5 kg of body weight leading to obesity (Rosen *et al.,* 1993).

2.4.6 Hypothalamic obesity

Obesity is common in patients with hypothalamic tumours, trauma, inflammation, or after hypothalamic surgery or radiotherapy (Apovian, 2016). Weight gain is thought to result from the injury of the ventromedial hypothalamic nucleus (Karam and McFarlane, 2007). The ventromedial hypothalamic nucleus' principal role is to integrate metabolic information regarding nutrient stores and food availability. Damage to these areas leads to hyperphagia, decreased metabolic rate, autonomic imbalance,

and GH deficiency, all of which result in progressive obesity (Pinkney *et al.,* 2002, Daousi *et al.,* 2005).

2.4.7 Hormone Ghrelin

Ghrelin is a potent appetite stimulant hormone produced in the stomach and stimulates food intake (Klok *et al.,*2007). Its levels are elevated 1 to 2 hours before a meal and then decrease (Apovian, 2016). Exogenous ghrelin is associated with increased food intake, reduced resting energy expenditure, and catabolism in adipose tissue (Moehlecke *et al.,* 2016). The consumption of excess calories may be linked to ghrelin. Ghrelin controls a person's level of hunger. When one is hungry, the hormonal levels of ghrelin increase and after eating the hormonal levelssubside (The Regents of the University of California, 2002). However, ghrelin is controlled by the intake of solid food and not liquids.Therefore, the consumption of SSB may not alleviate the symptoms of hunger. Consequently, the continued unchecked consumption of SSB results in an increased intake of calories which inevitably causes obesity (The Regents of the University of California, 2002).

2.4.8 Hormone Leptin

Leptin is released from fat cells located in the adipose tissue, and it transmits signals to the hypothalamus in the brain regarding energy availability and storage. Leptin can suppress appetite and increase energy expenditure resulting in weight loss (Morris and Rui, 2009). However, when leptin signalling is not functioning correctly it can result in weight gain or loss.

2.4.9 Pharmacotherapy

Several medication classes have been associated with weight gain, including antidepressants, anti-diabetic, antipsychotics, antiepileptic drugs, and beta-blockers (Karam and McFarlane, 2007). Many treatments for T2D are associated with weight gain with the potential of patients gaining up to 10 kg within six months after the start of treatment (Apovian *et al.,* 2015). It has been reported that weight gain of 1.7 to 3 kg occurred when consuming thiazolidinediones; sulfonylureas and insulin (Dailey *et al.,* 2010; Apovian *et al.,* 2015). The effect of insulin is usually associated with increased hunger and appears to be dose-dependent (Ness-Abramof and Apovian, 2005). A

potential explanation of the weight gain with insulin is the improved utilisation of calories through a decrease in glycosuria (Karam and McFarlane, 2007). Antipsychotics, antidepressants, and antiepileptics can increase body weight probably through their effect on the monoamines in the central nervous system (CNS) (Karam and McFarlane, 2007). Among newer neuroleptic medications, clozapine and olanzapine have been associated with an average weight gain ranging between 3 to 4.4 kg and an increased risk of diabetes and dyslipidemia (Newcomer *et al.,* 2007).

2.5 Genetic causes

One of the genetic contributions to BMI, namely, waist circumference, is thought to be related to susceptibility genes. Human obesity appears to be polygenic in the majority of cases (Karam and McFarlane, 2007). If both parents are obese, approximately 80% of their offspring will be obese, and if only one parent is obese the likelihood of obese offspring falls to 10% (*ibid.*). In a recent study, Li *et al.* (2007) reported that there were 12 obesity-susceptible loci. The investigators examined the association between those loci and BMI, waist circumference, weight, and height, as well as the predictive value for obesity risk (Li *et al.,*2007). Variants had an accumulative effect on obesity measures with each additional allele associated with an increase in weight of about 444 g and an increased risk of obesity of 10.8% (*ibid.*). However, the combined alleles had limited predictive value for obesity risk. Nonetheless, genetic influences on BMI appear to be strongly correlated (Stunkard *et al.,*1990; Price *et al.,* 1991).

Several genes such as pro-opiomelanocortin (*POMC*), prohormone convertase one and melanocortin four receptor (*MC4R*) are involved in signalling between external sites and the hypothalamic centre of satiety and hunger(Karam and McFarlane, 2007). In many cases, alterations within these genes lead to abnormal eating behaviours followed by the development of severe early-onset obesity (*ibid.*).

Severe obesity is a characteristic of many congenital and genetic disorders such as Alstrom–Hallgren syndrome, Bardet–Biedl syndrome, Beckwith–Wiedeman syndrome, Carpenter syndrome, Cohen syndrome and Prader–Willi syndrome (PWS), the latter being one of the most common syndromic forms of obesity in children (Rankinen *et al.,* 2001). In addition to being overweight, children with genetic syndromes associated with obesity typically have characteristic physical

abnormalities, including dysmorphic features, developmental delay and mental retardation (Karam and McFarlane, 2007).

2.6 The genes associated with obesity

Genes such as *ATP10A, CD44, WT1, MEG3,* and *TNFRS9* have been reported to play a role in obesity. This study investigated differentially methylation regions located in their respective CpG islands

2.6.1 *CD44* gene

A cluster of differentiation 44 (*CD44*) is a multifunctional cell membrane protein that can act as a receptor for hyaluronan and osteopontin and other biological processes (Johnson *et al.,* 2009; Toole, 2009). The *CD44* gene is expressed by most cells, including macrophages and hepatocytes, and has been implicated in many biological processes, including development, cancer metastasis, and cell adhesion (Johnson, 2009; Zoller, 2011; Liang *et al.,* 2011). During obesity, *CD44* expression is elevated in the liver and white adipose tissue (WAT) (Kang *et al.,* 2013). This suggests a possible regulatory role for *CD44* in metabolic syndrome (*ibid.*). The study by Kang (2013) suggested that *CD44* may play a critical role in linking obesity to the development of insulin resistance by promoting hepatic steatosis and the infiltration of macrophages in adipose tissue.

2.6.2 *MEG3* gene

Maternally expressed gene 3 (*MEG3*) is a maternally non-coding, imprinted gene that is found on chromosome 14q32.3 and was first identified as its mouse homolog *Gtl2* (Zhang *et al.,* 2010). The gene imprint is facilitated by the upstream intergenic differentially methylated region (IG-DMR) (Lin *et al.,* 2003). The expression of the gene occurs in a variety of healthy tissues.However, the expression has been lost in many human cancer cell lines and tumours (Zhou *et al.,* 2012). Twelve different isoforms of *MEG3* are generated by alternative splicing (Zhang *et al.,* 2010). Expression of *MEG3* can often be lost in cancer cells; it acts as a growth suppressor in tumour cells and activates *p53* which is a pituitary transcript variant and has been associated with inhibited cell proliferation. Studies in mice and sheep suggest that an upstream *IG-DMR* regulates the imprinting of the region. Studies have revealed that obese parents

were more likely to have children with altered methylation outcomes at multiple imprint regulatory regions when compared to children born to non-obese parents (Zhang *et al.,* 2010, Soubry *et al.,* 2016).

2.6.3 *ATP10A* gene

Adenosine triphosphosphatase 10A (*ATP10A*) is a putative promoter region of the P-locus Fat Associated ATPase (PFATP), containing CpG islands (Dhar *et al.* 2002). The localisation of *ATP10A* neighbours the maternally expressed gene in the imprinted domain and its unusual inheritance pattern of the obesity phenotype has suggested that *ATP10A* might be imprinted and associated with body fat type (Kayashima *et al.,*2003). PFATP is differentially expressed in humans and mouse tissues with predominant expression in the testis and lower levels of expression in adipose tissue and other organs (Dhar *et al.,* 2000). It is also known to modulate body fat and lipid metabolism.

2.6.4 The *TNFRSF* gene

The tumour necrosis factor receptor superfamily (*TNFRSF*) is a cluster of cytokine receptors characterised by the ability to bind to a pro-inflammatory cytokine called the tumour necrosis factors (TNFs) via an extracellular cysteine-rich domain. It is also associated with systemic inflammation (Beutler and Cerami, 1988). *TNF*-α is produced by activated macrophages and can induce specific immune responses, including fever (Dinarello, 2004) and cell death (Sidoti-de Fraisse *et al.,* 1998). Elevated levels of *TNF*-α have been reported in obese patients and those with peripheral insulin resistance.*TNFRSF9* is overexpressed in obesity(Hotamisligil *et al.,* 1993). The increase does not only occur in circulating *TNF-α* levels but also in adipose tissue (Hotamisligil *et al.* 1995). The involvement of *TNF-α* in obesity has also been confirmed in animal studies (Uysal *et al.,* 1997).

2.6.5 The *WT1* gene

The Wilms' tumour 1 (*WT1*) protein plays a role in cell growth, cell differentiation, and the self-destruction of cells (apoptosis) (Makrigiannakis *et al.* 2001). To carry out these functions, the *WT1* protein regulates the activity of other genes by binding to specific regions of DNA. The *WT1* protein is called a transcription factor. A recent study

showed that most visceral fat arises from cells expressing the Wilms' tumour 1 (*WT1*) gene late in mouse gestation(Chau and Hastie, 2015).Visceral fat is the type of body fat found around the abdominal cavity. The cells that keep on expressing *WT1* continue to act as a source of visceral fat into adulthood when they may be influenced by external factors such as diet. Individuals that have central obesity, which is associated with a higher risk of obesity-related diseases including type 2 diabetes, cardiovascular diseases and cancer, tend to have more visceral adipose tissue (Chau and Hastie, 2015).

2.7 Epigenetics

2.7.1 The history of epigenetics

The role played by genes in human development was initially misunderstood. A majority of embryologists believed that genes only played insignificant roles in human development (Herrera *et al.,* 2011). However, Dr Waddington disagreed with that notion (*ibid.*). As highlighted by Herrera *et al.* (2011), Dr Conrad Waddington, was a British biologist, geneticist and philosopher, who laid the foundation for epigenetics in 1940. He believed that genes and their regulation via an epigenetic landscape were key towards controlling cell fate and how cells become specialised. Waddington also developed the related notion of genetic canalisation—the process whereby a trait becomes buffered against allelic heterogeneity. The term epigenetics describes the concept itself, as 'epi' means above or over in Greek (Waddington*,* 1940).

2.7.2 The definition of epigenetics

Epigenetics refers to natural control mechanisms that influence gene expression (Herrera *et al.,* 2011). It can also be defined as the covalent modification of DNA, RNA and proteins that results in a change of the function or regulation of the molecule without changing their primary sequence (Bird,2002). Epigenetic processes comprise of gene silencing, reprogramming, imprinting, X chromosome inactivation and carcinogenesis. In mammals, a vital cell function regulated by epigenetic processes is cell differentiation wherein during embryogenesis stem cells are entirely differentiated (Heyn *et al.,*2013; Tammen *et al.,*2013; Vidaki *et al.,*2013). The evidence to support the epigenetic concept was provided three decades later by Holliday and Pugh (1975)

when they identified DNA methylation as the first mechanism of epigenetics. Moreover, DNA methylation provided some explanation to X-chromosome inactivation and gene imprinting (Chen *et al.,* 2005). During the lifespan of an organism, epigenetic mechanisms provide an association between environmental factors and phenotypic changes (Figure 2.5).

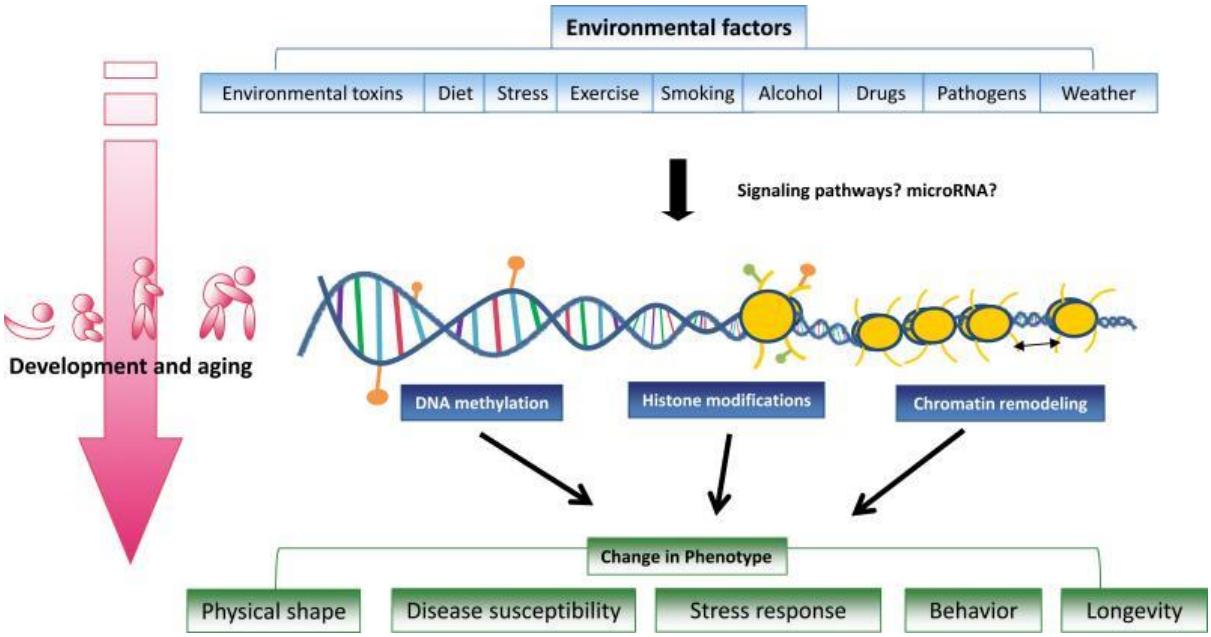

Figure 0.5: The association of epigenetic processes and phenotypic changes throughout an organism's lifespan (Tammen *et al.* 2013)

2.8 DNA methylation

DNA methylation is one mechanism that may result in an epigenetic effect. During DNA methylation the addition of a methyl group (CH_3) to cytosine occurs and covalently modifies the function of the gene (Herrera *et al.,* 2011). The most well-known DNA methylation process is the covalent addition of the methyl group at the 5-carbon of the cytosine ring resulting in 5-methylcytosine (5-mC) (Moore *et al.,* 2012). Cytosine methylation occurs when the GC dinucleotides are adjacent which results in two methylated cytosine residues sitting diagonal to each other on opposing DNA strands. According to Arzenani (2009), Hotchkiss first reported methylated cytosine in 1948 by using paper chromatography. Cytosines found within the cytosine phosphate guanine (CpG) rich regions tend to be the most methylated (Moore *et al.,* 2012).

2.8.1 CpG islands

These CpG islands are often found in or near the promoter regions of various genes (Illingworth and Bird, 2009). CpG islands can be located in the transcription start site in three ways as shown in Figure 2.6 (Srivastava *et al.,* 2018). These islands are termed CpGs because they are regions in a genome which have a higher than expected frequency of CG nucleotides (*ibid.*). These conspicuous unique sequences are approximately 1 kb in length and overlap the promoter regions of 60–70% of all human genes (Bird *et al.,* 1985; Larsen *et al.,* 1992; Lander *et al.,* 2001; Saxonov *et al.,* 2006).

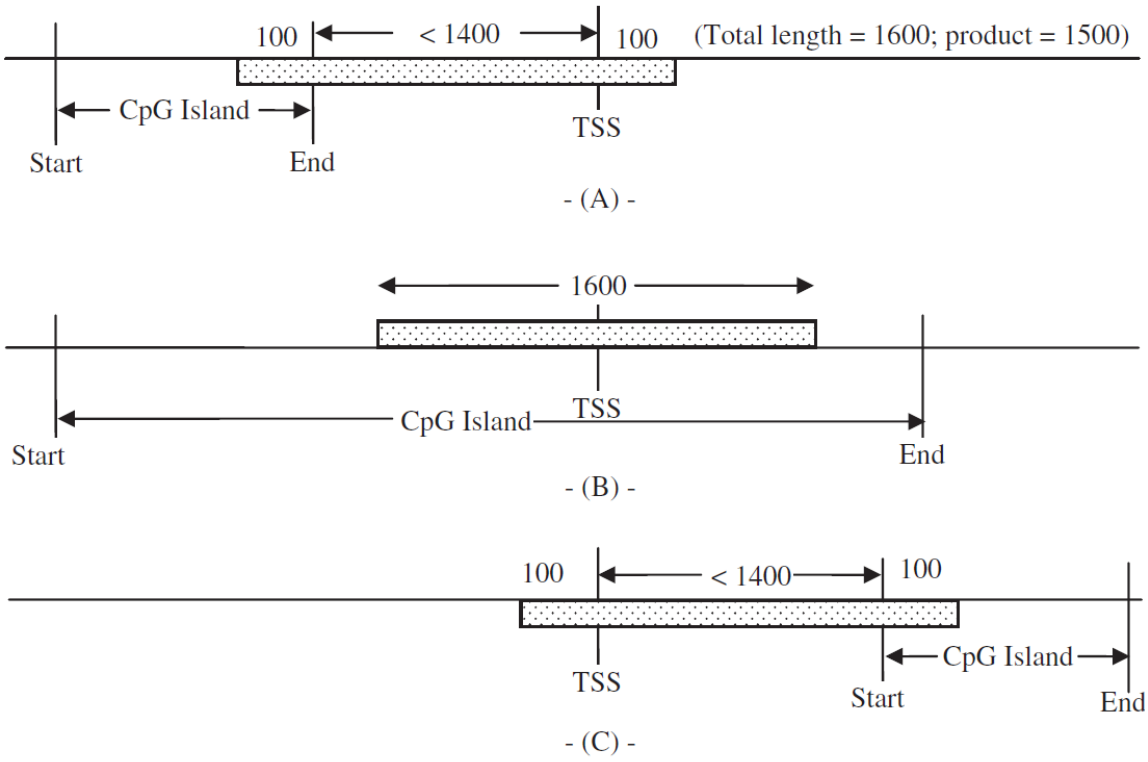

Figure 0.6: Three ways CpG islands can be located (Adapted from Srivastava *et al.,* 2018)

When the CpG Islands in the promoter region of a gene have methylated the expression of the gene is repressed (Bird,2002). Gene repression, in this case, is achieved by obstructing access to transcription factors/activators (Bird, 2002). The recruitment of co-repressors such as histone deacetylases which may alter the structure of the chromatin results in the failure to initiate transcription (Herrera *et al.,* 2011) as shown in Figure 2.7 following.

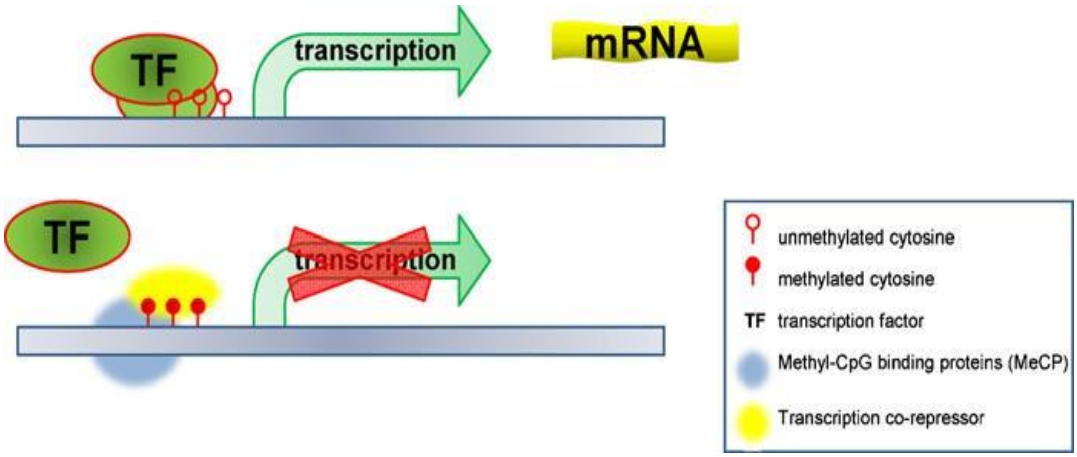

Figure 0.7: CpG methylation and regulation of gene expression.[Unmethylated or hypomethylated DNA (usually in the promoter region) allows binding of the transcription factors (TF) and other regulatory mechanisms which result in transcription and mRNA production. Methylated DNA (bottom panel) obstructs binding of the TF and in some cases might recruit methyl-CpG binding proteins and other transcription co-repressors, blocking access of the transcription enzymes and resulting in gene silencing] Source: Herrera *et al.,* 2011)

Unmethylated or hypomethylated DNA within the promoter region allows for the binding of the transcription factors (TF) which results in the transcription of mRNA Methylated DNA, on the other hand, blocks access to the TFs as well as enzymes within the promoter region involved in the transcription process thus resulting in gene silencing (Herrera *et al.,* 2011).

2.8.2 Enzymes involved in DNA methylation

DNA methylation in eukaryotic cells is mediated by two types of DNA methyltransferases (DNMT); these are DNMT1 and DNMT3 (Thompson *et al.* 2010). DNMT1 is responsible for methylation during DNA synthesis, whereas DNMT3a and DNMT3b are responsible for the maintenance of methylated regions of DNA following DNA replication (Thompson *et al.,* 2010). These enzymes are essential in mammalian development. They contribute to two enzymatic activities which are sustaining methylation post-replication and *de novo* methylation to institute new DNA methylation patterns throughout early development (Lalruatfela, 2013; Kader and Ghai, 2015; Zampieri *et a,l.* 2015).

2.8.3 Environmental factors influencing DNA methylation

Although DNA methylation occurs naturally, it may be influenced by environmental factors (Campion 2009). Increasing evidence has demonstrated that environmental and lifestyle factors may influence epigenetic mechanisms.The influences are apparent in DNA methylation patterns (Hunter, 2005; Alegria-Torres *et al.,*2011; Tammen *et al.,*2013). Studies have demonstrated a link between DNA methylation and nutrient intake as critical factors in obesity (Campion, 2009). It has been demonstrated in epigenetics and obesity research projects that factors like nutrient intake, diet and conditions such as inflammation, oxidative stress and hypoxia can affect the DNA methylation of an individual as well (Campion, 2009). Various dietary bioactive food components have been observed to alter gene expression via changes inDNA methylation (Hardy and Tollefsbol, 2011; Park *et al.,* 2011). Besides the dietary effect, hypo- and hyper-methylation have been associated with ageing (Bird,2003; Fraser *et al.,* 2012; Day *et al.,* 2013; Tammen *et al.,* 2013). With an increase in age, DNA methylation machinery tends to lose its ability to maintain methylation patterns across cellular divisions (Goyal *et al.,* 2006).

2.9 Methods used to study DNA methylation

2.9.1 Bisulfite conversion

Bisulfite conversion has been used to assess the methylation profile of genes (Frommer,1992). During bisulfite conversion, the genomic DNA changes from large and stable double-stranded molecules into randomly fragmented single-stranded molecules.Bisulfite conversion involves the deamination of the unmethylated cytosine to uracil, leaving 5-mC or 5-hmC intact (*ibid.*) (Figure 2.8). Uracil is then amplified in subsequent PCR reactions as thymine, whereas 5-mC or 5-hmC residues get amplified as cytosine (Huang, 2010).

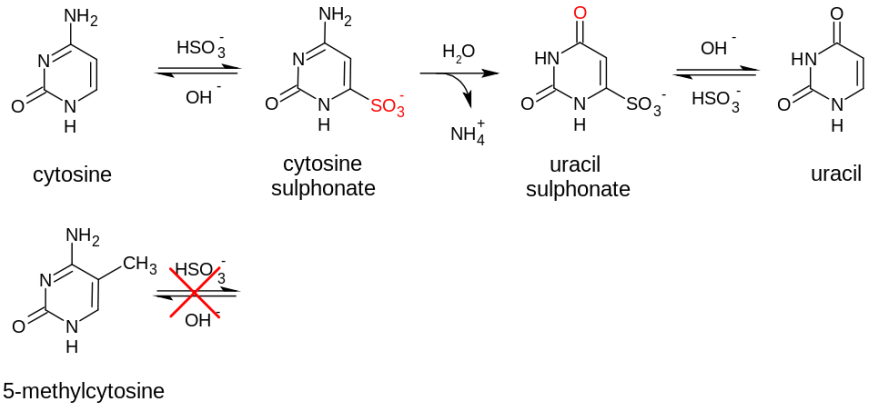

Figure 0.8: Chemical reaction for the bisulfite conversion of Cytosine to Uracil, whilst the 5-methylcytosine remains intact after the reaction (Source: Applied Biosystems, 2007)

2.9.1.1 Bisulfite conversion PCR

Bisulfite genomic sequencing is regarded as a gold-standard technology for the detection of DNA methylation because it provides a qualitative, quantitative and efficient approach to identify 5-methylcytosine at the single base-pair resolution (Li and Tollefsbol, 2011). This method was first introduced by Frommer *et al.* (1992), and it is based on the finding that the amination reactions of cytosine and 5- methylcytosine (5mC) proceed with very different consequences after the treatment of sodium bisulfite. The cytosine bases in single-stranded DNA are converted to uracil residues and recognised as thymine in subsequent PCR amplification and sequencing. However, 5mCs are unchanged in this conversion and remain as cytosines allowing 5mCs to be distinguished from unmethylated cytosines (Hayatsu, 1970). A subsequent PCR process is done to determine the methylation status in the loci of interest by using specific methylation primers after the bisulfite treatment (Li and Tollefsbol, 2011). The actual methylation status can be determined either through direct PCR product sequencing (detection of average methylation status) or sub-cloning sequencing (detection of single molecules distribution of methylation patterns). Moreover, bisulfite sequencing analysis can not only identify DNA methylation status along the DNA single-strand but also detect the DNA methylation patterns of DNA double strands since the converted DNA strands are no longer self-complementary, and the amplification products can be measured individually (Li and Tollefsbol, 2011).

2.9.2 MALDI-TOF Mass spectrometry

Matrix-Assisted Laser Desorption Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) offers high throughput identification of methylation sites and semi-quantitative measurement of their single or multiple CpG locations (Stassen *et al.* 2004; Ehrich *et al.* 2005). The conversion of unmethylated cytosine to uracil during bisulfite treatment will generate base-specific cleavage products that reflect underlying methylation patterns that can be readily analysed by MALDI-TOF MS (Milagro *et al.,* 2011).

2.9.3 Restriction enzymes to detect methylation

The restriction digestion method to detect methylation relies on the use of isoschizomer enzymes, i.e. *Hpa*II and *Msp*I (Frommer, 1992). These enzymes recognise a similar palindromic sequence; however, they exhibit different sensitivities to methylated DNA (*ibid.*). The ability of these enzymes to discriminate between methylated and unmethylated DNA sequences makes them invaluable in epigenetic studies.

2.9.4 Pyrosequencing

Pyrosequencing is a real-time sequencing method used to analyse the bisulfite converted, amplified DNA, for DNA methylation profiling (Shen *et al.,*2007). The method screens the incorporation of nucleotides via the conversion of releasing pyrophosphate into a relatively light signal. The visible light is generated and may be viewed as peaks in a pyrogram. The heights of peaks are indicative of the number of identical nucleotides that are integrated into the sequence; a double peak would imply that two nucleotides were added. Degree of CpG methylation is software-calculated from the ratio of thymine to cytosine (Tost and Gut, 2007; Madi *et al.,*2012).

2.10 Caco-2 cell line as a model cell line for the epigenetic study

The study of epigenetic events *in vitro* requires the use of ideal cell lines. The use of cell lines, such as *Ca*ncer *co*li-2 (Caco-2),started in 1970 (Sambuy *et al.,* 2005). Caco-2 was established from a human colorectal adenocarcinoma at the Sloan-Kettering Cancer Research Institute. Caco-2 cells were used to investigate the mechanism for

cancer development (Fogh *et al.,* 1977). They were used due to the heterogeneity of primary intestinal epithelial cells in morphology and functions (Table 2.1). Several of the cell lines could be partly differentiated by the addition of synthetic or biological factors to the medium (Verhoeckx*et al.,* 2015). However, one of them, Caco-2, was unique as it was able to differentiate spontaneously when reaching confluence (*ibid.*).

Table 0.1: Properties of Caco-2 cells (Source:Verhoeckx*et al.,* 2015)

Attribute	Comment
Growth	Grows in culture as an adherent monolayer of epithelial cells
Differentiation	Takes 14–21 days after confluence under standard culture conditions
Cell morphology	Polarised cells with tight junctions and brush border at the apical side
Electrical parameters	High electrical resistance
Digestive enzymes	Expresses typical digestive enzymes, membrane peptidases and disaccharidases of the small intestine (lactase, aminopeptidase N, sucrase-isomaltase and dipeptidyl-peptidase IV)
Active transport	Amino acids, sugars, vitamins, hormones
Membrane ionic transport	Na^+/K^+ ATPase, H^+/K^+ ATPase, Na^+/H^+ exchange, $Na^+/K^+/Cl^-$ cotransport, apical Cl^- channels
Membranenon-ionic transporters	Permeability glycoprotein (P-gp, multidrug resistance protein), multidrug resistance-associated protein, lung cancer-associated resistance protein
Receptors	Vitamin B12, vitamin D3, EGFR (epidermal growth factor receptor), sugar transporters (GLUT1, GLUT2, GLUT3, GLUT5, SGLT 1)
Cytokine production	IL-6, IL-8, TNFα, TGF-β1, thymic stromal lymphopoietin (TSLP), IL-15

The Caco-2 cell line can ideally be used to investigate dietary factors like sugar sweeteners that may result in an epigenetic event. It undergoes a culture of spontaneous differentiation leading to the formation of a monolayer of cells expressing morphological and functional characteristics of a mature enterocyte (Sambuy *et al.,*

2005). The Caco-2 cell lines exhibit a monolayer of viable, polarised and fully differentiated villus cells similar to those found in the small intestines (Menier *et al.,* 1995). The Caco-2 cell line has a high proliferation potential resulting in a cell population (Natoli *et al.,* 2012). In culture they form tight junctions, spontaneously differentiate, express relatively high levels of digestive brush border enzymes and display other morphological, structural and functional properties similar to intestinal enterocytes (Pinto *et al.,* 1983).

2.11 Cytotoxicity assay

2.11.1 LDH assay

The Lactose dehydrogenase (LDH) assay measures the release of the LDH enzyme from the cytoplasm of cells into the cell medium. Its evaluation of cytotoxicity is based on the decrease of membrane integrity to retain the LDH in the cytoplasm when cells are exposed to membrane-damaging toxic agents (Silva et al., 2010). LDH assay possesses characteristics such as speed, simple evaluation and reliability. Its popularity has risen in research the recent years, due to these qualities.

2.11.2 Alamar Blue assay

AlamarBlue is a cell viability assay reagent which contains the cell-permeable, non-toxic, and weakly fluorescent blue indicator dye called resazurin (O'Brien, 2000)The assay is a quantitative cytotoxicity test used to measure the proliferation of various human and animal cell lines, bacteria and fungi. The bioassay may also be used to establish the relative cytotoxicity of agents within various chemical classes (Fields *et al.,* 1993). This method owes its extensive use in research to its homogeneity and provision of the high sensitivity of detection of cell densities as low as 200 cells/well (Page *et al.,* 1993).

2.12 Statistical terms used in this study

The cytotoxic effects of the different concentrations of sugar sweeteners and incubation time on cell viability were evaluated using Analysis of Variance (ANOVA). It is a statistical tool used to detect differences between the experimental group by comparing the means of different groups and showing if there are any statistical

differences between the means (Sawyer, 2009). P-value, F- value and F critical value are used to measure statistical significance. A p-value is a measure of the probability that an observed difference could have occurred just by random chance. F-value is a measure used to show if the variance between populations is significantly different. F-critical value is a tool that compares the combined effect of all the variables together. When F value in a test is larger than your F-critical value, you can reject the null hypothesis**.** The varibles have a statistical significance.A p-value less than 0.05 (typically ≤ 0.05) is statistically significant. Supposing an ANOVA does not yield statistical significance on any main effects or interactions; in that case, the Null hypothesis is accepted, meaning that the different levels of independent variables did not have any differential effects on the dependent variable (Sawyer, 2009). In the case that the ANOVA yields statistical significance on the main effect or interactions;in that instance, the null hypothesis is rejected, and the alternative hypothesis is accepted, meaning that the different levels of independent variables have any differential effects on the dependent variable.

CHAPTER THREE

RESEARCH DESIGN AND METHODOLOGY

3.1 Research design

The research for this study followed the design shown in Figure3.1 below.

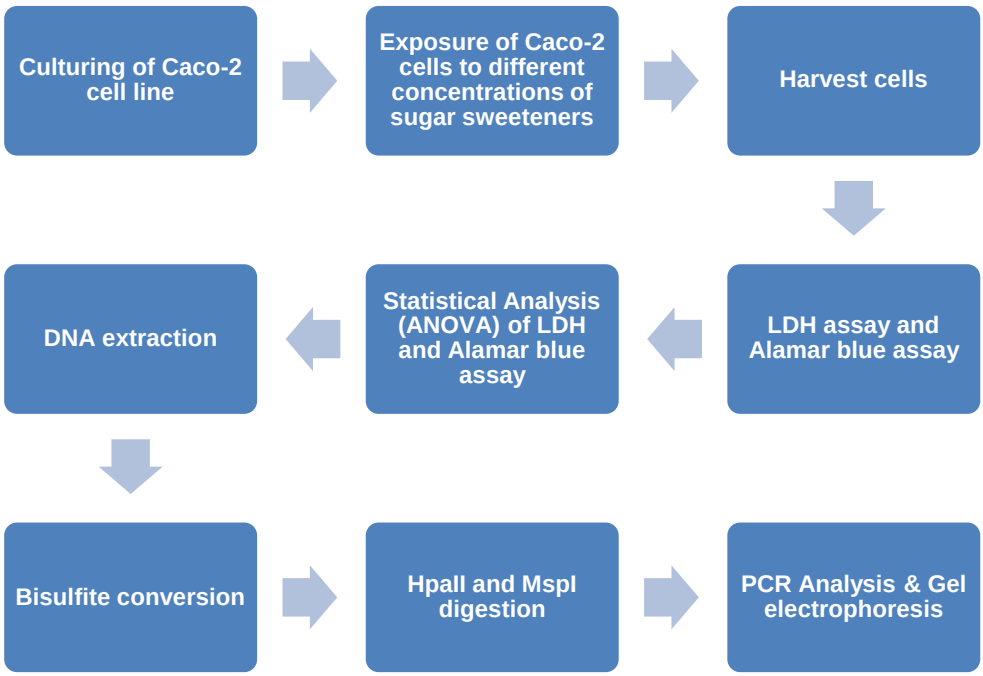

Figure 3.1: The research design

3.2 Culturing of the caco-2 cell line

A human colorectal adenocarcinoma cell line (Caco-2) from Cellonex (Johannesburg, South Africa) was cultured and maintained in Dulbecco's Modified Eagle Minimum Essential Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin solution and 1% non-essential amino acids at 37ºC in a five percent (5%)carbon dioxide (CO_2)atmosphere incubator (ESCO, Horsham, PA). The culture medium was replaced with fresh DMEM media every two to three days until the cells were 80% confluent. The confluent cells were washed thrice with Dulbecco's Phosphate Buffer Saline (DPBS) and then trypsinised. For the neutralisation of Trypsin-EDTA solution, an equal volume DPBS and 10% FBS was added. Thereafter the cell count was determined with a Countess™ Automated cell counter (Invitrogen, Eugene, OR).

3.3 Stimulation of the caco-2 cells with different concentrations of sugarsweeteners and morphological characterisation of the cells

This study used fructose and glucose as sweeteners since popular beverages have a fructose: glucose ratio of approximately 60:40 (Walker *et al.* 2014). Onemolar stock solutions of fructose and glucose were prepared separately in 50 mL of ultra-pure water. The stock solutions were used to prepare a low, medium and high concentration of sweeteners, maintaining a fructose and glucose ratio of 60:40 (Table 3.1).

Table 0.1 Mixtures of glucose and fructose representing low, medium and high concentrations utilised in this study

Type of Concentration	Fructose concentration	Amount of stock solution added (fructose)	Glucose concentration	Amount of stock solution added(Glucose)	Final volume
Low	0.17 M	0.85 mL	0.11M	0.55mL	5 mL
Medium	0.3 M	1.5 mL	0.2 M	1 mL	5 mL
High	0.5.M	2 mL	0.3 M	1.5 mL	5 mL

The low, medium and high sugar solutions (Table 3.2) were used to culture the Caco-2cells in 15 mL conical tubes in triplicate.Volumes of 3.6 mL, 2.5 mL and 1.5 mL of DMEM were added into the low, medium and high concentration tubes, respectively to a final volume of 5 mL. After every 4th day, the media was replaced with fresh media supplemented with the low, medium and high sugar solutions. Cells that were exposed for 12, 20, 32, 42, 52, and 62 days to the sugar solutions were then trypsinised for three minutes and resuspended in media. Approximately 80% of the cells (4 x10^5 cell/mL) were plated in a 12-well plate for the continuation of the sugar sweeteners treatment experiment and 20% of the cells (2 x10^5 cell/mL) were harvested and stored at -80°C for DNA extraction.

3.3.1 Morphological characterisation of the cells

After the exposure of the Caco-2 cells to different concentrations of the sugar sweeteners for 1, 4, 8, 12, 20, 32, 42, 52, and 62 days, images of the cells were captured using an inverted IX53 microscope (Olympus, Tokyo, Japan).

3.4 Evaluation of the cytotoxicity of different concentrations of the sugar sweetener on the caco-2 cell line

3.4.1 Alamar blue percentage viability assay

A 96-well plate seeded at a cell density of 2 x10^5 cells/mL was used for the cytotoxicity assay.Cell viability was determined using the Alamar blue assay (Invitrogen, Eugene, OR) following the manufacturer's instructions. The media of the cells that were incubated for one, four and eight days was replaced with 100 µL of fresh media. Then, 20 µL of Alamar blue solution was added into each well. The plates were covered with foil and incubated for four hours at 37°C. The absorbance was measured at wavelengths of 570 nm and 600 nm using Epoch plate reader (PROMEGA, Sunnyvale, CA), and the percentage cell viability was calculated using equation 1 below:

$$\%\ cell\ viability = \frac{(\mathbf{O2\ x\ A1}) - (\mathbf{O1\ x\ A})}{(O2\ x\ A1) - (O1\ x\ P2)} x100$$

- O1 = molar extinction coefficient (E) of oxidized Alamar Blue (blue) at 570 nm*
- O2 = E of oxidised Alamar Blue at 600 nm*
- A1 = absorbance of test wells at 570 nm
- A2 = absorbance of test wells at 600 nm
- P1 = absorbance of positive growth control well (cells plus Alamar Blue but no test agent) at 570 nm
- P2 = absorbance of positive growth control well (cells plus Alamar Blue but no test agent) at 600 nm

3.4.2 LDH assay

After one, four and eight days of treatment of the Caco-2 cells with the low, medium and high sugar concentrations, the LDH assay (Thermo Fischer, Rockford, IL) was performed following the manufacturer's instructions. As controls, four wells of untreated cells were added with 10 µL of sterile water (spontaneous LDH) and the other four wells containing untreated cells, were added with 10 µL of the 10X lysis buffer. After the addition of water and lysis buffer, the 96-well plate was incubated at 37°C for 45 minutes. Following the incubation, 50 µL of the solution from each well

was transferred to a new 96-well plate and 50 µL of the reaction mixture was added to each well. The plate was covered with foil and incubated for 30 minutes at room temperature. Thereafter, the reaction was stopped by adding 50 µL of the stop solution to each well. The absorbance was measured at wavelengths of 490 nm and 680 nm using Epoch plate reader (PROMEGA) and the percentage cytotoxicity was calculated using equation 2:

$$\% \text{ Cytotoxicity} = \frac{\text{Compound−treated LDH activity − Spontaneous LDH activity}}{\text{Maximum LDH activity − Spontaneous LDH activity}} \text{ x100}$$

3.4.3 Statistical analysis

The data for the cytotoxicity test was analysed using ANOVA with a statistical significance set at $p < 0.05$. ANOVA was conducted using the Prism GraphPad (version 7).

3.5 DNA methylation studies

3.5.1 DNA extraction from Caco-2 cells

DNA was extracted from the treated Caco-2 cells using the Thermo Scientific GeneJET™ Genomic DNA Purification kit according to the manufacturer's instructions. The Caco-2 cells were centrifuged at 8000 × g for five minutes. The supernatant was decanted, and the pellet was rinsed once with 50 µL Phosphate Buffer Solution (PBS) to remove the residual medium. The pellet was then resuspended in 200 µL of PBS, to which 200 µL of the lysis buffer and 20 µL of Proteinase K solutions were added. The mixture was vortexed to obtain a uniform suspension followed by ten minutes incubation at 56°C in a shaking water bath. Thereafter, 20 µL of RNAse A (10 µg/mL) solution was added to the sample, followed by ten minutes incubation at 21°C. Then, 400 µL of 50% ethanol was added followed by a brief vortex. The lysates were transferred into a purification column that was inserted in a collection tube. The column was centrifuged for one minute at 6000 × g and the collection tube was discarded together with the filtrate. The column was then inserted into a new collection tube and 500 µL of wash buffer 1 was added followed by centrifugation for one minute at 6000 × g with the filtrate being discarded thereafter. After this, 500 µL of wash buffer 2 was added into the column followed by

centrifugation for three minutes at 13 300 × g. The collection tube with the filtrate was discarded. The column was inserted into a 1.5 mL sterile centrifuge tube and 200 µL of the elution buffer was added to the centre of the column membrane. The preparation was incubated at 21°C for two minutes and centrifuged for one minute at 8000 × g. The concentration of the DNA was determined using a Nanodrop™ UV/VIS spectrophotometer (using 1 OD_{260}= 50 µg ds DNA). The purified DNA was stored at -80°C until used.

3.5.2 Bisulfite conversion

Genomic DNA (50 ng) from the experimental samples were subjected to sodium bisulfite conversion using the New England Biolabs® inc (New England Biolabs, Ipswich, MA) kit according to the manufacturer's instructions. A mixture consisting of genomic DNA extracted from the experimental samples of the different concentrations and untreated Caco-2 cells together with EpiMark® bisulfite mix was transferred to a thermocycler T100 Bio-Rad Thermocycler (Bio-Rad). Bisulfite conversion in the thermocycler was performed for three hours following the manufacturer's instructions.

After completion of the bisulfite conversion, the DNA Binding Buffer was added to each sample followed by a brief vortex. Then each sample was transferred onto an EpiMark spin column with a collection tube attached. The columns were centrifuged at 15,000 x g for one minute and the flow-through was discarded. Wash Buffer was added to each EpiMark spin column and the sample centrifuged at 15,000 × g for one minute and the flow-through was discarded. Thereafter, the desulphonation reaction buffer was added to each column and incubated at room temperature (18–20°C) for 15 min. The columns were centrifuged at 15,000 × g for one minute and the flow-through was discarded. Wash Buffer was added, the columns were centrifuged at 15,000 × g for one minute, and the filtrate was discarded. The wash step was repeated twice. The spin columns were placed into sterile 1.5 mL microcentrifuge tubes, Elution Buffer was added and incubated for one minute at room temperature. The columns were centrifuged at 15,000 x g for one minute. Elution Buffer was again added, and tubes were centrifuged for 30 seconds at 15,000 x g. The DNA was ready for methylation analysis by PCR.

3.5.3 Primer design

PCR primers were designed to complement the bisulfite converted DNA. The design was developed with the aid of the MethPrimer tool found at (www.urogene.org). The primers were designed based on the following parameters: (i) product size:150-300 bp; (ii) primer size: 26-30bp; (iii) annealing temperature: 55-60ºC; GC content: 30-40%; and (v) should not contain any CpG site where methylation varies.

The expected results of the PCR with the bisulfite converted DNA were not obtained although several attempts were made at the bisulfite conversion process. Therefore, a different strategy was adopted for further research. New primers were then designed,using Bisearch tool,to amplify the CpG islands of the five obesity-related genes using untreated DNA. The primers were designed based on the following parameters: (i) product size: 200-400 bp; (ii) primer size: 18-35 bp; (iii) annealing temperature: 59-64ºC; and(vi) GC content: 50-60%. The primers (Table 3.2) were synthesized by Inqaba Biotechnical Industries, Pretoria, South Africa.

Table 0.2: The primer sequences used for bisulfite-treated DNA and untreated DNA

Name of gene	Primer Sequence 5'-3'	Type of DNA
CD44 gene	Forward primer: 5'-AAGTATTATTTGGTTGAAAGAAAAAGAGA-3' Reverse primer: AAAATTATAATCCCTTCTATAACCACC	Bisulfite
	Forward primer GAAAGCCCACCTCAGAGAAT (50164) Reverse primer: CCTTCATGCTCCAATTCACC (50397)	Unconverted
MEG 3 gene	Forward primer: GAGAGTAGGTAAAGTTAAGATTTTGGAA (81957-81963) Reverse primer: CAAACCCCTAACATAAAAATTCCTAC (82291-82266)	Bisulfite
	Forward primer: CCTTCCTGCTTCTGATTTCTCC (17090) Reverse primer: GCCCTTCATAAACCTGTTC (17478)	Unconverted
ATP10A gene	Forward primer: bisulfite TTATGATGTGGAGGGGAGTTT (3987-4007) Reverse primer: AAATATCTATTTCCCTCCCTAAAAA (4187-4163)	Bisulfite
	Forward primer: normal CCGGCCTTTATCAAGCATTC (95660) Reverse primer: GCCTACGTCACTCATTTCAG (96058)	Unconverted
WT1 gene	Forward primer: AAGTTAAGTAGAAAGGTGGTTTGGG (3285-33100 Reverse primer: AACTTTCCAACCAAACTTATATAAAACAA (3572-3544)	Bisulfite
	Forward primer: normal CAGGAAAAACTGGTGATTTGG (27395) Reverse primer: AGTAAAGGGAGAAGGAGGAG (27674)	Unconverted
TNFSRF9 gene	Forward primer: GTAGATTTAGGGTTAAGGGAAGTT (5502-5525) Reverse primer: ACCTACCCATCATTAAACAACTAAA (5901-5877)	Bisulfite
	Forward primer CTGGTTGCTAGTGAGGTTAG (17116) Reverse primer: ATTTAGTAGGCTGAGGCAGG (1750)	Unconverted

3.5.4 PCR analysis

3.5.4.1 PCR amplification for the bisulfite-treated DNA

The primer sequences for the bisulfite converted DNA are shown in Table 3.3. About 30 ng of bisulfite converted DNA was used for each PCR. Reactions (50 µL) contained 25 µL of 2x PCR GoTaq® HotStart Mastermix (Promega, WI, USA), 5 µL of each forward and reverse primer (10 µM), 4 µL DNA template, 4mM of $MgCl_2$ and 11 µL of Nuclease-free water. The PCR mixture was placed in a T100 Bio-Rad Thermocycler (Bio-Rad, Hercules, CA). The thermal cycling conditions used are shown in Table 3.3: The amplicons were run on a 1% gel in a Bio-Rad electrophoresis system for 45 minutes at 120 V in a 1xTBE buffer. The images of the gels were captured in a Bio-Rad Gel Doc™ EZ Imager using ImageLab™ Software version 5.0.

Table 0.3: PCR cycling condition used for the bisulfite-treated DNA

Cycle Step	**Temperature (°C)**	**Time**
Initial Denaturation	95	30 seconds
40 cycles	95	15 seconds
Elongation	54-60 (Gradient mode due to different primer temperatures) *WT1:* 59.4 *ATP10A:*58.4 *CD44:* 59.5 *MEG3:*59.5 *TNFSRF9*: 55.3	30 seconds
	72	1 min/kb
Final Extension	72	5 min

3.5.4.2 PCR amplification for the untreated DNA

Standard PCR was performed using the second set of primers. A 50 µL reaction mixture, containing 25 µL of 2x PCR GoTaq® HotStart Mastermix (Promega), 5 µL of each forward and reverse primer (10 µM), 4 µL DNA template, 4mM of $MgCl_2$ and 11 µL of Nuclease-free water. The reaction mixture was placed in a T100 Bio-Rad Thermocycler (Bio-Rad). The thermal cycling conditions used are shown in Table 3.4. The size of fragments was confirmed by visualisation on 1% agarose gels stained with

ethidium bromide. The images of the gels were captured in a Bio-Rad Gel Doc™ EZ Imager using ImageLab™ Software version 5.0.

Table 0.4: PCR cycling condition for the standard PCR

Cycle Step	**Temperature (°C)**	**Time**
Initial Denaturation	95	30 seconds
40 cycles Elongation	95	15 seconds
	(Gradient mode due to different primer temperatures) WT1: 62 ATP10A: 60 CD44: 61.5 MEG3: 63.4 TNFSRF9: 59	30 seconds
	72	1 min/kb
Final Extension	72	5 min

3.5.5 Methylation sensitive restriction enzymes digestion

Restriction enzyme digestion of the extracted DNA was performed using the New England Biolabs cut smart restriction digestion kit according to the manufacturer's instructions. Two methylation-sensitive enzymes were used, namely *Hpa*I and *MspII.* For *Hpa*I and *Hpa*I digestion, 5 µL (0.1–0.5 µg) of PCR product was added directly to a mixture containing 1 µL of the enzyme, 5 µL of cutsmart buffer, and 9 µL of nuclease-free water in respective tubes The tubes were centrifuged for 16 seconds at 5000 ×g, followed by incubation overnight at 37°C. The amplicons were separated on a 1% agarose gels stained with ethidium bromide.

CHAPTER FOUR

RESULTS

4.1 Morphological characterisation of the caco-2 cell line

4.1.1 Morphological characterisation of the Caco-2 cell line after 1day exposureto sugars of different concentrations

The morphology of the cells was characterised after one day of treatment with the sweeteners at a low, medium, and high concentration using a microscope (Please note: Day one was just after the cells were transferred into a 96-wells plate, where the treatment with the sugar sweeteners took place).As controls, the cells were either untreated or treated with 50% hydrogen peroxide. The untreated cells, 70% confluent were attached close to each other forming an intact monolayer (Figure. 4.1a) while the cells treated with 50% hydrogen peroxide (H_2O_2) (Figure.4.1b) appeared small and round and have decreased in numbers. The cells treated with the low concentration of the sweeteners attained cumulus shapes and were semi spreading. Relative to the untreated cells, they were dissociating from each other, and a disrupted monolayer was forming (Figure 4.1c and d). Cells treated with medium and high (Figure 4.1g and h) sweetener concentrations showed similar morphological characteristics. However, round-shaped cells had drifted away and detached from the walls and floating in the media were also observed.

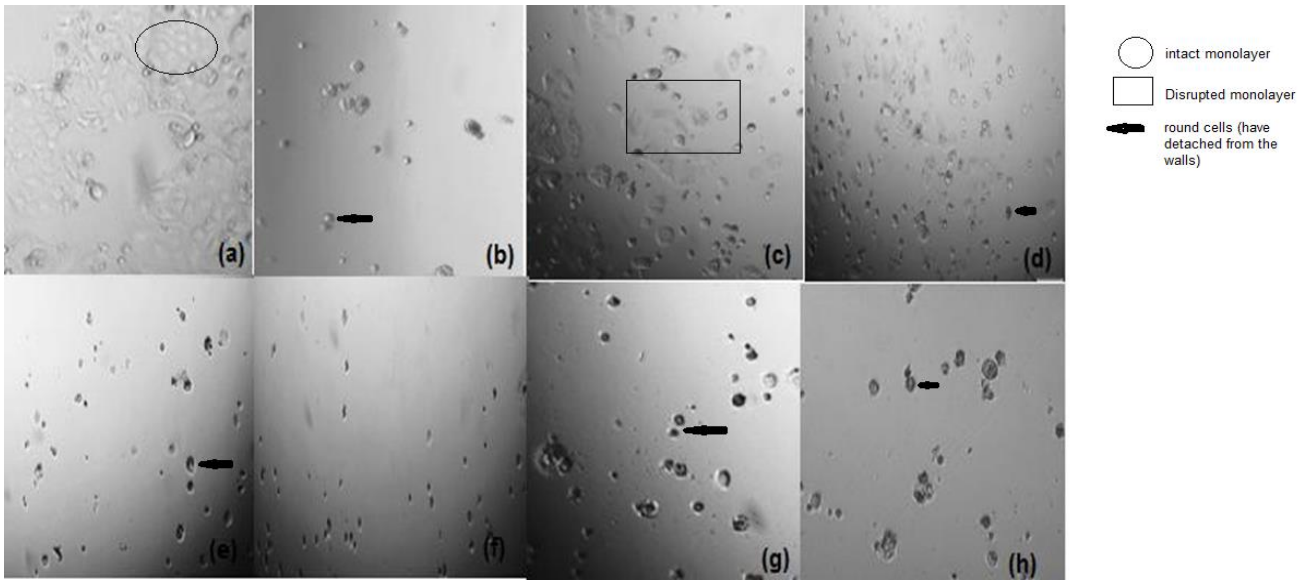

Figure 0.1: (a) Caco-2 cells treated with fresh culture media as a negative control (b)Cells treated with 50 % H_2O_2 as a positive control, (c and d) Cells treatedtreated with a low concentration,(e and f) Cell treated with medium concentration and (g and h) Cells treated with high concentration of the sugars sweeteners incubated for one day, respectively. Magnification 4x.

4.1.2 Morphological characterisation of the Caco-2 cell line after four days of exposure to sugars of different concentrations

After four days of exposure, the untreated cells appeared to be 95% confluent in the wells, had increased in numbers, showing an intact monolayer that is attached to the wells and the cells had formed tighter junctions. The cells that were treated with 50% hydrogen peroxide (H_2O_2) had decreased in number and had a clump of round cells floating (Figure4.2b). Cells treated with a low sugar concentration were rounded and attached to the wells. Some of the cells had dissociated from each other, forming a disrupted monolayer and had a rough appearance (Figure4.2c-d). Cells treated with a medium (Figure4.2e-f) and high (Figure4.2g-h) concentrations showed a similar morphology. In both concentrations, the cells appeared round. There was a dissociation of cells followed by dark clumps of dead cells floating in the medium. The number of cells treated with high concentration had decreased.

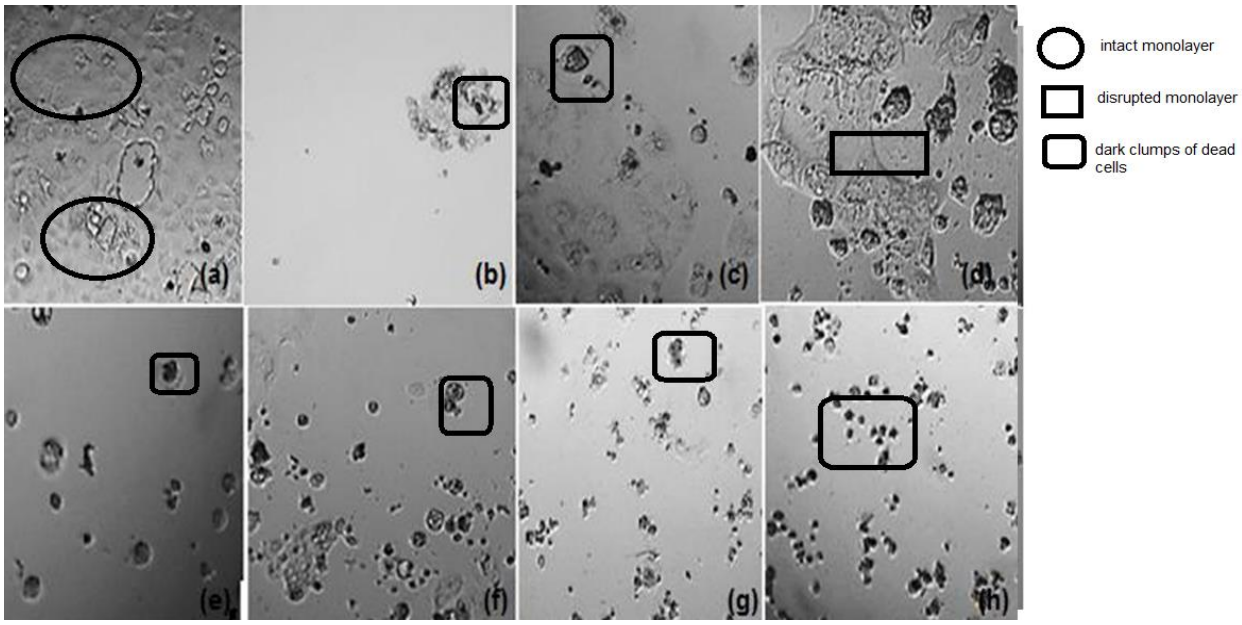

Figure 0.2: (a) Caco-2 cells treated with fresh culture media as a negative control (b) Cells treated with 50 % H_2O_2 as a positive control, (c and d) Cells treated with a low concentration,(e and f) Cell treated with medium concentration and (g and h) Cells treated with high concentration of the sugars sweeteners incubated for four day, respectively. Magnification 4x.

4.1.3. Morphological characterisation of the Caco-2 cell line after eight days of exposure to different concentrations of sugar sweeteners

After eight days of exposure, the untreated cells appeared to be 100% confluent in the wells (Figure4.3). All the cells had differentiated into monolayer, forming the tight junction of cells and they were still attached to the well. The cells treated with 50% hydrogen peroxide (H_2O_2) were dead and floating in the medium. Low concentration, compared to the untreated cells, some of the cells had dissociated from each other, forming disrupted monolayers and single cells (Figure4.3c-d). Cells treated with a medium (Figure4.3e-f) and a high (Figure4.3g-h) concentrations were similar in morphology. The cells treated with these concentrations had decreased in number, they were varied in size but were mostly small and round, some cells had formed dark clumps of dead cells.

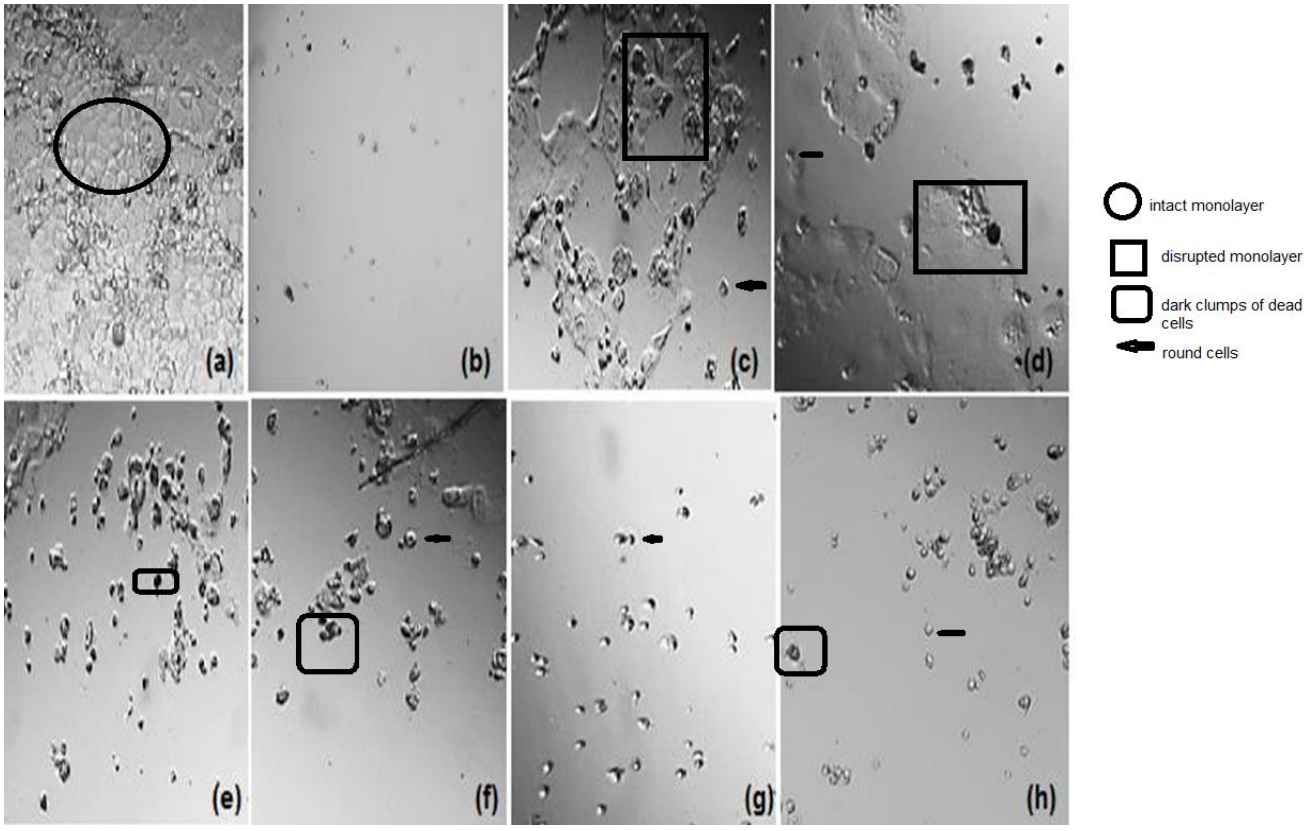

Figure 0.3: (a) Caco-2 cells treated with fresh culture media as a negative control (b) Cells treated with50 % H_2O_2 as a positive control, (c and d) Cells treated with a low concentration,(e and f) Cells treated withmedium concentration and (g and h) Cells treated with high concentration of the sugars sweeteners incubated for eight day, respectively. Magnification 4x.

After eight days of exposure, the treatment of the cells with different concentrations continued; the experiment was carried out to effect an epigenetic change by

prolonging the exposure of the cells to sweeteners. Upon observing that the high concentration resulted in a decrease of cell viability and cell density, it was omitted from the test. There were continuous intervals of nutrient renewal. After days 12, 20,32,42,52 and 62 of experimental exposure, cells were harvested, and some cells were re-seeded for the experiment to continue.

4.1.4 Morphological characterisation after 12 days of exposure to different concentrations of sugar sweeteners

Cells treated with 50% hydrogen peroxide (positive control) were dead and floating in the medium (figure 4.4a). The untreated cells (negative control) appeared round and varied in size, and the cells seemed 100 % confluent and were closed attached characterising an intact monolayer (Figure4.4b).The cells treated with the low concentration of the sugar sweeteners appeared to be irregular in shape, varied in size and were also close to each other (Figure 4.4c). The cells treated with the medium concentration were also round with varying sizes, and a few cells were elongated. The cells were scattered and had dissociated from each other (Figure 4.4d).

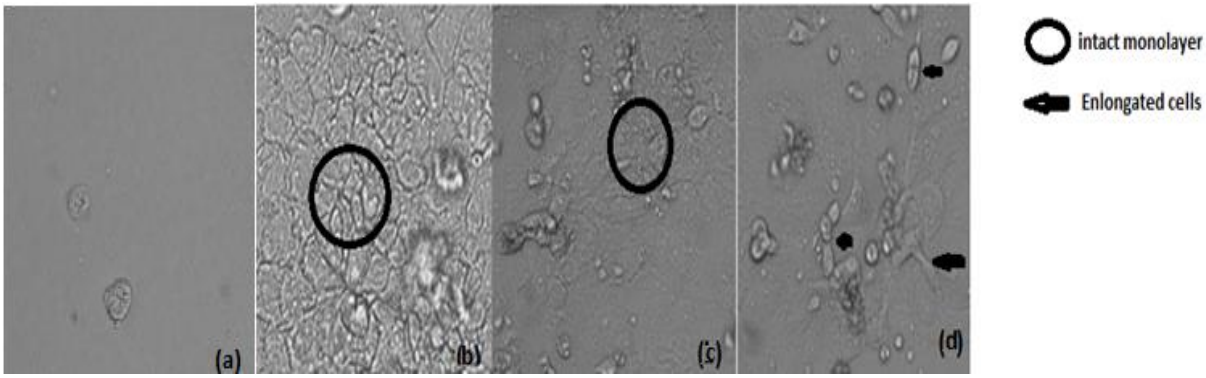

Figure 0.4: (a) Caco-2 cells treated 50% hydrogen peroxide, (b)Caco-2 cells treatedwith fresh culture media as a negative control, (c) Cells treated with a low concentration and (d) Cells treated with medium concentration of the sugar sweeteners incubated for 12 days, respectively. Magnification 20×.

4.1.5 Morphological characterisation after 20 days of exposure to different concentrations of sugar sweeteners

Cells treated with 50% hydrogen peroxide (positive control) were dead and floating in the medium (figure 4.4a). The untreated cells appeared to be variable in size, and the cells were tightly close to each other forming an intact monolayer; there were cell debris floating and clumps of cells. The cells were confluent (Figure 4.5b).The cells

treated with the low concentration of the sugars appeared to be round, and they varied in size and had decreased in cell density. Some cells had dissociated from each other, forming a disrupted monolayer, and some cells were still attached (Figure 4.5c).

The cells treated with the medium concentration were also round. Some of the cells were starting to form tapering ends. A decrease in cell density (Figure 4.5d) was also observed.

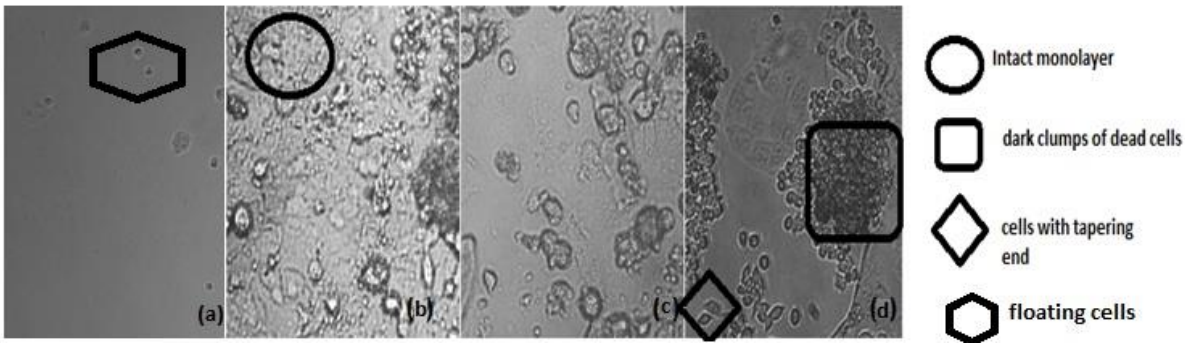

Figure 0.5: (a) Caco-2 cells treated 50% hydrogen peroxide, (b) Caco-2 cells treatedwith fresh culture media as a negative control, (c) Cells treated with a low concentration and (d) Cells treated withmedium concentration of the sugar sweeteners incubated for 20 days, respectively. Magnification 20×.

4.1.6 Morphological characterisation after 32 days of exposure to different concentrations of sugar sweeteners

Cells in fresh culture media were used as a negative control (untreated cells). The morphology of the treated cells was viewed under a Nikon microscope at 20x magnification (Figure4.6a–d). Cells treated with 50% hydrogen peroxide (positive control) were floating in the medium(Figure 4.6a).The untreated cells (negative control) appeared to be over confluent. The cells had to form multiple layers onto each other increasing cell density (Figure 4.6b). The cells had formed an intact monolayer.The cells treated with the low concentration of the sugar sweeteners appeared round, were smaller in size, and there were large clumps of dead cells. Some cells were close to each other, and others had dissociated from each other (Figure 4.6c).The cells treated with the medium concentration were varying in shape and size, and some of the cells had the tapering ends. Some cells were floating in the medium resulting in a decrease in cell density (Figure 4.6d).

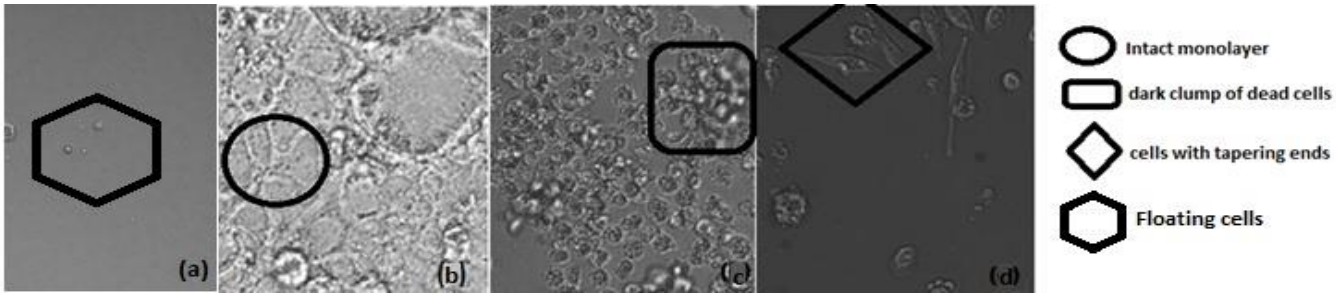

Figure 0.6: (a) Caco-2 cells treated 50% hydrogen peroxide, (b) Caco-2 cells treatedwith fresh culture media as a negative control, (c) Cells treated with a low concentration and (d) Cells treated withmedium concentration of the sugar sweeteners incubated for 32 days, respectively. Magnification 20×.

4.1.7 Morphological characterisation after 42 days of exposure to different concentrations of sugar sweeteners

Cells infresh culture media were used as a negative control (untreated cells). The treated cells were incubated for 42 days, and the morphology of the cells was visualised under a Nikon microscope at 20x magnification (Figure4.7a – d).Cells treated with 50% hydrogen peroxide (positive control) were dead and no longer viable(Figure 4.7a). The untreated cells (negative control) were over confluent the surface of the wells, formed layers of cells on top of each other displaying an intact monolayer, and there was a large clump of cellular mass not attached to the walls of the well (Figure 4.7b).The cells treated with the low concentration of the sugar sweeteners appeared to be round, and they varied in size and had decreased in cell density, the cells were not close to each other, and there was also cell debris floating (Figure 4.7c). The cells treated with the medium concentration had changed shape, displaying abnormalities in morphology. The cells were irregular in shape with the tapering ends.There was cell shrinkage and vacuolation (Figure 4.7d)

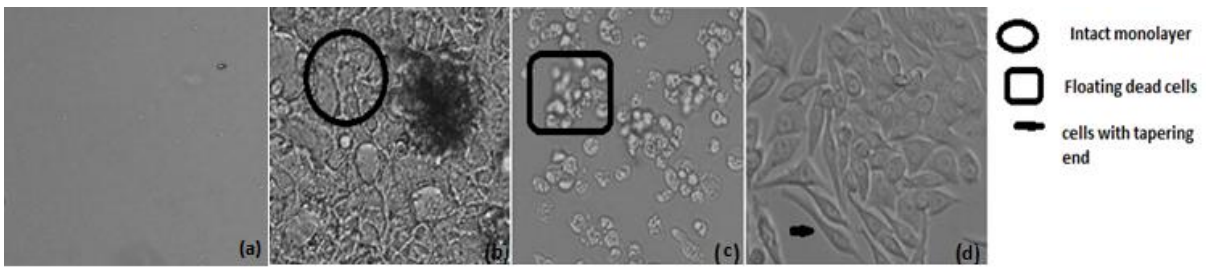

Figure 0.7: (a) Caco-2 cells treated 50% hydrogen peroxide, (b) Caco-2 cells treatedwith fresh culture media as a negative control, (c) Cells treated with a low concentration and (d) Cells treated withmedium concentration of the sugar sweeteners incubated for 42 days, respectively. Magnification 20×.

4.1.8 Morphological characterisation after 52 days of exposure to different concentrations of sugar sweeteners

At day 52 of incubation, the morphology of the cells was visualised under a Nikon microscope at 20x magnification (Figure4.8a – d). Cells treated with fresh culture media were used as a negative control (untreated cells).Cells treated with 50% hydrogen peroxide (positive control) were dead and no longer viable (Figure 4.7a). The untreated cells (negative control) over confluent, formed layers of cells on top of each other and had increased in cell density (Figure 4.8b).The cells treated with the low concentration of the sugar sweeteners appeared to be round, and they varied in size.There was a large aggregated clump of cell mass floating (Figure 4.8c).The cells treated with the medium concentration had changed shape, displaying abnormalities in morphology. The cells had irregular shapes, with some cells being round and some with tapering ends, and there was cell shrinkage as well (Figure 4.8d)

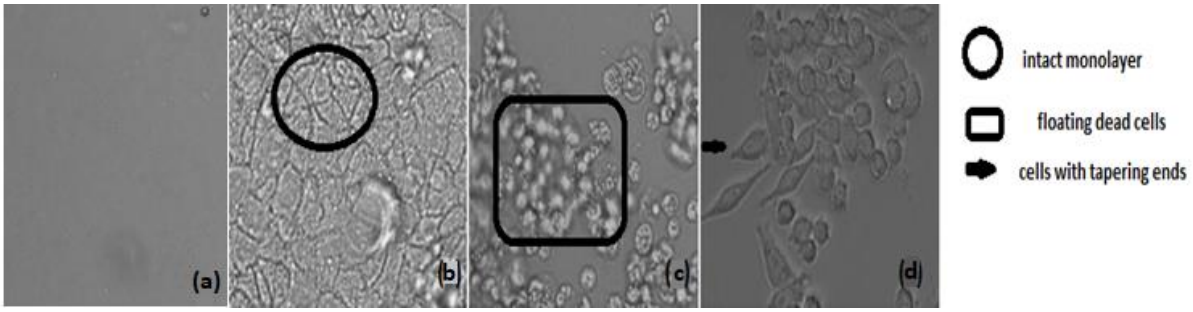

Figure 0.8: (a) Caco-2 cells treated 50% hydrogen peroxide, (b) Caco-2 cells treatedwith fresh culture media as a negative control, (c) Cells treated with a low concentration and (d) Cells treated withmedium concentration of the sugar sweeteners incubated for 52 days, respectively. Magnification 20×.

4.1.9 Morphological characterisation after 62 days of exposure to different concentrations of sugar sweeteners

After 62 days of incubation, the morphology of the cells was visualised under a Nikon microscope at 20x magnification (Figure4.9a – d). Cells treated withfresh culture media were used as a negative control (untreated cells).Cells treated with 50% hydrogen peroxide (positive control) were dead andfloating in the medium (Figure 4.7a). The untreated cells (negative control) were over confluent, forming intact monolayers of cells on top of each other, and some cells were dying, forming large

clumps of floating dead cells (Figure4.9b).The cells treated with the low concentration of the sugar sweeteners appeared to be changing shape as well; they were no longer round but varied in size forming cells with tapering ends. There was a large aggregated clump of cell mass floating (Figure4.9c).The cells treated with the medium concentration had decreased in cell density.The cells displayed some morphological abnormalities; cell shrinkage had occurred,and most cells had died (Figure4.9d).

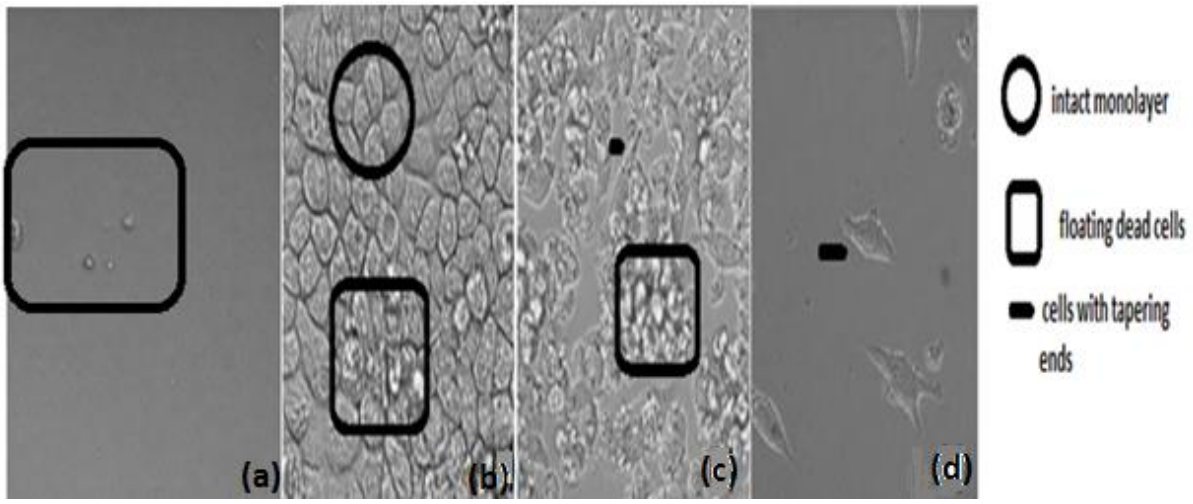

Figure 0.9: (a) Caco-2 cells treated 50% hydrogen peroxide, (b) Caco-2 cells treatedwith fresh culture media as a negative control, (c) Cells treated with a low concentration and (d) Cells treated withmedium concentration of the sugar sweeteners incubated for 62 days, respectively. Magnification 20×.

4.2 Cytotoxicity studieson caco-2 cells

4.2.1 Effect of sweeteners on the cell viability of Caco-2 cells

Assessment of cell viability was performed using the Alamar blue assay, that evaluated the effect of different concentrations of sugar sweeteners on the metabolic activity of the Caco-2 cells over time (Please note that glucose and fructose were used as a combination and the terms low, medium and high concentration are used to describe the type of concentrations).

The results showed that there was a decrease in the number of metabolically active cells across all treatments with time. When the cells were exposed to a low concentration of the sugar sweeteners, metabolic activity relative to the untreated cells which were at 100% was reduced to 20± 1.26 %, 25± 2.26 % and 45 ± 3.91 % after one, four and eight days of incubation, respectively (Figure 4.10). The cells exposed

to the medium concentration of the sugar sweeteners showed reduced metabolic activity relative to the untreated cells at an average of 25± 0.48 %, 40± 0.85 % and 65± 1.37 % after one, four and eight days of incubation, respectively (Figure4.10). Cells treated with the high concentration of the sugar sweeteners showed reduced metabolic activity to an average of 26± 0.11 %, 60± 0.85 %, 75 ± 1.56 % after one, four and eight days of incubation, respectively (Figure4.10). The cells treated with hydrogen peroxide (H_2O_2) (positive control), showed a decrease of 95± 0.09 %, 99 ± 0.89% and 100 ± 0.89% in metabolic activity, after one, four and eight days, respectively (Figure4.10).

The results for the Alamar blue assay illustrated that cell viability was dependent on both concentration and the incubation time as shown in Figure 4.10.The number of viable cells was inversely proportional to sugar sweetener concentration and incubation time (Figure 4.10).

The results showed that the metabolic activity of the cells is directly proportional to cell viability. Cells treated with the low concentration of the sugar sweeteners had a cell viability of 80%, 75% and 55% after one, four and eight days, respectively (Figure4.10). Cells treated with the medium concentration of sugar sweeteners had a cell viability of 75%, 60% and 35% after one, four and eight days, respectively (Figure4.10). Cells treated with the medium concentration of sweeteners had a cell viability of 74%, 40% and 25% after one, four and eight days, respectively. Cells treated with medium and high concentrations after four and eight days were less metabolically active thus indicating an abrupt decrease in cell viability as compared to the cells treated with low concentration at that time point.

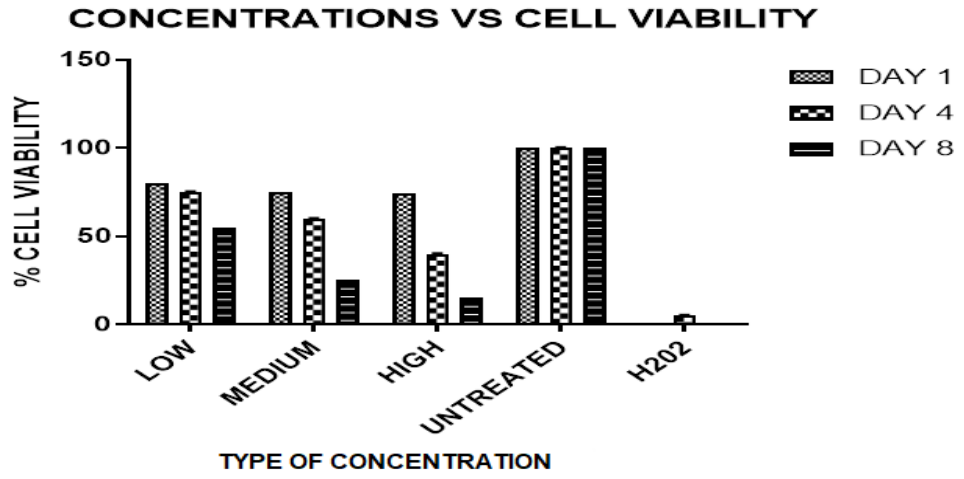

Figure 0.10: Cell viability (%) of Caco-2 cell line after treatment with low (0.11 M of glucose and 0.17 M of fructose), medium (0.2 M of glucose and 0.3 M of fructose), high (0.3 M of glucose and 0.5 M of fructose) concentrations of the sweeteners and hydrogen peroxide concentration using Alamar blue assay.

4.2.2 ANOVA report of the effect of the sugar sweeteners on cell viability of Caco-2 cells.

The effects of the different concentrations of sugar sweeteners and incubation time on cell viability were compared using ANOVA. Concentration had a p-value of 0.033, and the number of days had a P-value of 0.0134. According to the results of the p-value, a statistically significant ($p>0.05$) was found between the two groups when cell viability is considered. The concentration had a F-value of 4.87587 and a F-critical value of 4.45897. According to the results ($F_{value} > F_{critical}$), there was a stastical significance. The number of days had a F-value of 4.4024 and a F-critical value of 3.837853. According to the results ($F_{value} > F_{critical}$), there was a stastical significance.

Table 0.1: ANOVA report of the effect of the sugar sweeteners on cell viability of Caco-2 cells.

Source of Variation	Degree of freedom	Sum of Squares	Mean Square	F-value	P-value	F critical value
Rows (concentration)	2	138,1333	69,06667	4.87587	0,033123	4,45897
Columns (number of days)	4	2494,4	623,6	4,4024	0.0134	3,837853
Error	8	1133,2	141,65			
Total	14	3765,733				

4.2.3 LDH cytotoxicity assay

LDH assay was used to assess the LDH release from a cell quantitatively.The results showed that there was an increase in the level of LDH released across all treatments with time (Figure4.11). The results obtained from cells treated with low concentration relative to the positive control after one day showed that LDH leakage increased by 22.49 ± 0.33 %, 37.85 ± 1.03 %, and 51 ± 0.75 % after one, four and eight days of incubation, respectively (Figure4.11). Cells exposed to the medium concentration of the sweeteners had an increase in LDH leakage of 27.88± 33 %, 45.21± 1.26 %,

65.47± 0.95 % after one, four and eight days, respectively (Figure4.11). The cells treated with the high concentration of the sugar sweeteners on average had an increase in LDH release of 29.33% ± 0.18, 51.36± 0.75 % and 70.25 ± 1.27% after one, four and eight days, respectively (Figure.4.11). The untreated cells had an average of 8.28± 0.63 %, 11.87± 1.27 %and 15.53 % ± 0.12 LDH leakage after one, four and eight days, respectively (Figure4.11).

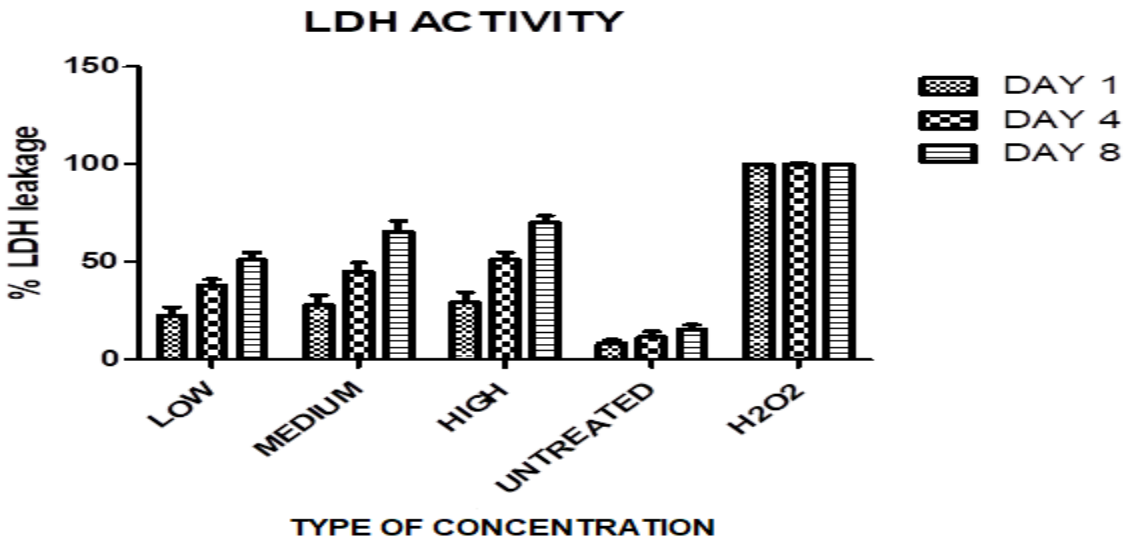

Figure 0.11: % of LDH leakage of Caco-2 cell line after treatment with low (0.11 M of glucose and 0.17 M of fructose), medium (0.2 M of glucose and 0.3 M of fructose), high (0.3 M of glucose and 0.5 M of fructose) concentrations of the sweeteners and hydrogen peroxide concentration using LDH assay

4.2.4 ANOVA report of the cytotoxic effect of the different concentrations of sugar sweeteners on cell viability of Caco-2 cells

The ANOVA showed that the data consisted of significant ($p < 0.05$) model terms. The ANOVA shows that the concentration ($p=0.0231$), as well as the number of days of incubation ($p=0.0357$), were statistically significant. (Table 4.2). The concentration had a F-value of 5.0547 and a F-critical value of 4,4634.According to the results ($F_{value} > F_{critical}$), there was a stastical significance. The number of days had a F-value of 4,48421 and a F-critical value of 3,678. According to the results ($F_{value} > F_{critical}$), there was a stastical significance

Table 0.2: ANOVA report of the cytotoxic effect of the different concentrations of sugar sweeteners on cell viability of Caco-2 cells.

Source of Variation	Degree of freedom	Sum of Squares	Mean Square	F-value	P-value	F critical value

Rows (concentration)	2	131.309	65.66	5.0547	0,023123	4,4634
Columns (number of days)	4	1241.4	310.3	4,48421	0,035714	3,678
Residual (random)	8	678	8476			
Total	14	3765,733				

4.3 DNA methylation studies

4.3.1Bisulfite PCR

For methylation studies on the CpG islands, bisulfite primers were designed, and the DNA obtained from the experimental samples was bisulfite converted. There was no amplification from the bisulfite-treated DNA from day 12-62, no bands on the gel for each of the five obesity-related genes (results notshown). No amplification can be due to various reasons, some of which are discussed in Chapter 5.

4.3.2 PCR analysis for untreated DNA

The genomic DNA was amplified using the primers mentioned in Table 3.5. The primers were designed to amplify the CpG islands of the genes. PCR fragments were visuralised on a one percent (1%) agarose gel stained with ethidium bromide.

4.3.2.1 PCR Analysis after 12 days of incubation

Day 12 for Caco-2 cells treated with a low concentration of sugar sweeteners resulted in clear bands for the *CD44*, WT1, ATP10A, *TNFRSF9,* and the MEG genes (Figure4.12.). The molecular weight for the WT1 was 390 bp, ATP10A was 399 bp, TNFSRF9 was 390 bp, and *MEG3* genes were approximately 389 bp.Only the *CD44* gene had a low molecular weight of approximately 234 bp. Amplification for Caco-2 cell line treated with a medium concentration (Figure4.12b.) of the sugar sweeteners

displayed clear bands for all the genes amplified (*CD44*, WT1, ATP10A, *TNFRSF9.*

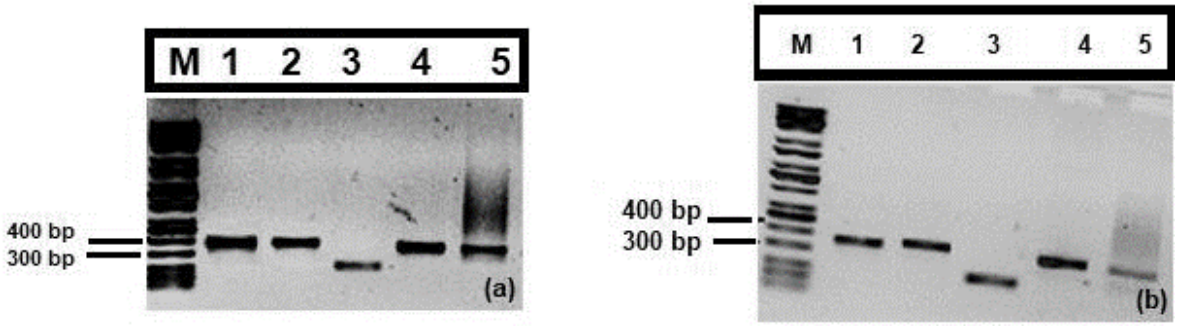

Figure 0.12: Figure 4.12: PCR amplification of the five obesity-related genes from the Caco-2 cell line treated with a low (a) and medium concentration (b) on day 12. PCR fragments were visualised on a 1 % agarose gel stained with ethidium bromide. Lanes represent the following: M. KAPA Universal Ladder, 1. *WT1*, 2. ATP10A, 3. *CD44*, 4. *TNFRSF9,* and 5. *MEG3.*

4.3.2.2. PCR Analysis after 20 days of incubation

PCR amplification at day 20 appeared to be similar to data obtained in day 12, for Caco-2 cells treated with a low concentration of sugar sweeteners resulted in clear bands for the *CD44*, *WT1*, ATP10A, *TNFRSF9* and the MEG genes (Figure 4.13). The molecular weightz for the *WT1* was 390 bp, *ATP10A* was 399 bp, *TNFSRF9* was 390 bp, and *MEG3* genes were approximately 389 bp.Only the *CD44* gene had a low molecular weight of approximately 234 bp. Amplification for Caco-2 cell line treated with a medium concentration of the sugar sweeteners displayed clear bands for all the genes that were amplified (*CD44*, *WT1*, *ATP10A*, *TNFRSF9,* and the *MEG*3 genes). The molecular weight for the *WT1*, ATP10A, TNFSRF9, and *MEG3* genes were approximately 390 bp.The *CD44* gene had a low molecular weight of about 250 bp. The *MEG3* gene displayed a smear (Figure 4.13).

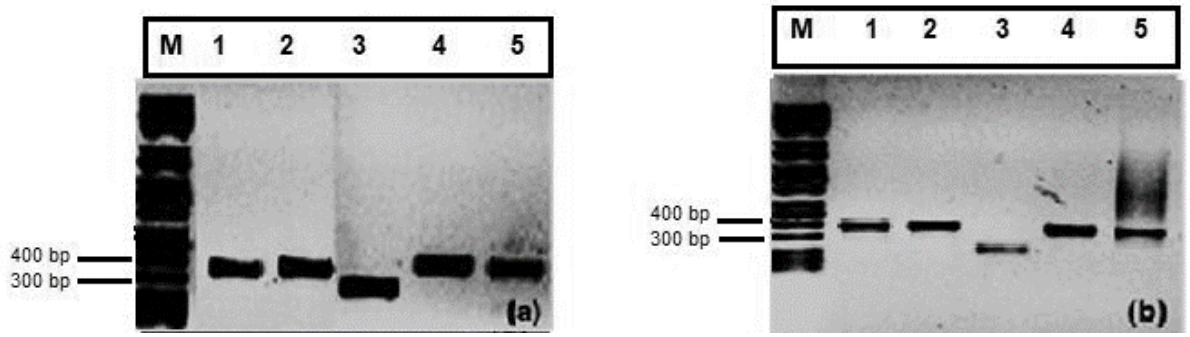

Figure 0.13: PCR amplification of the five obesity-related genes from the Caco-2 cell line treated with a low concentration (a) and medium concentration (b) at day 20. PCR fragments were visualised on a 1% agarose gel stained with ethidium bromide. Lanes represent the following: M. KAPA Universal Ladder, 1. *WT1*, 2. *ATP10A*, 3. *CD44*, 4. *TNFRSF9,* and 5. *MEG3*.

4.3.2.3 PCR Analysis after 32 days of incubation

Amplification at day 32 for Caco-2 cells treated with a low concentration of sugar sweeteners resulted in clear bands for the *CD44*, *WT1*, *ATP10A*, and *TNFRSF9*. The *MEG3* appeared to have a band and a smear (Figure4.14). The molecular weight for the *WT1* was 390 bp, *ATP10A* was 399 bp, *TNFSRF9* was 390 bp, and *MEG3* genes were approximately 389 bp.*CD44* gene had a low molecular weight of approximately 234 bp. Amplification for Caco-2 cell line treated with a medium concentration of the sugar sweeteners displayed clear bands for the genes that were amplified (*CD44*, *ATP10A*, *TNFRSF9*), whereas the *WT1* had an unclear band and the *MEG3* gene had a smear and a band.

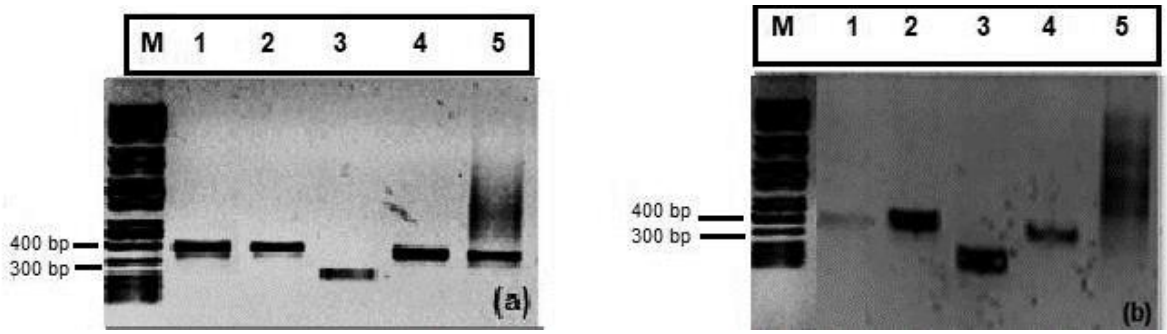

Figure 0.14: PCR amplification of the five obesity-related genes from the Caco-2 cell line treated with a low concentration (a) and medium concentration (b) at day 32. PCR fragments were visualised on a 1% agarose gel stained with ethidium bromide. Lanes represent the following: M. KAPA Universal Ladder, 1. *WT1*, 2. *ATP10A*, 3. *CD44*, 4. *TNFRSF9,* and 5. *MEG3.*

4.3.2.4 PCR Analysis after 42 days of incubation

Amplification at day 42 for Caco-2 cells treated with a low concentration of sugar sweeteners resulted in clear bands of the *CD44*, *WT1*, *ATP10A*, and *TNFRSF9*. The *MEG3* appeared to have a band and a smear (Figure4.15). The molecular weight for the *WT1* was 390 bp, *ATP10A* was 399 bp, *TNFSRF9* was 390 bp, and *MEG3* genes were approximately 389 bp while the *CD44* gene had a low molecular weight of approximately 234 bp. Amplification for Caco-2 cell line treated with a medium concentration of the sugar sweeteners displayed clear bands for the genes that were

amplified (*CD44*, *ATP10A*, *TNFRSF9*), whereas the *MEG3* gene had a smear and a band (Figure 4.15).

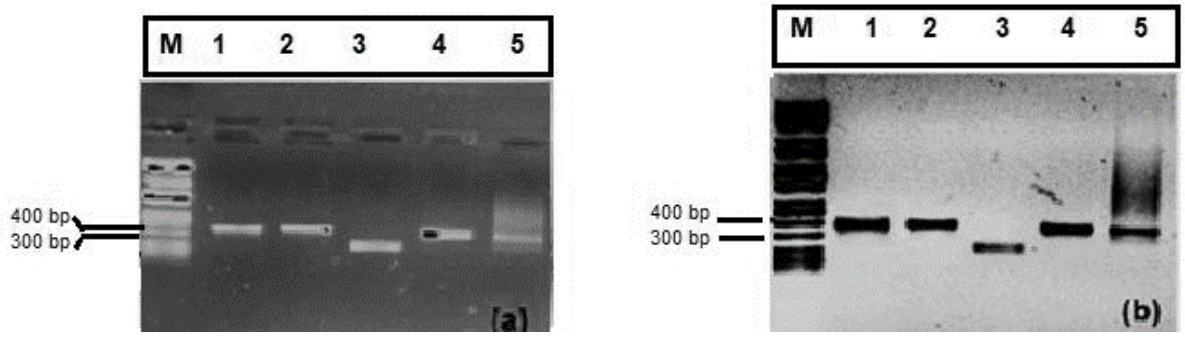

Figure 0.15: PCR amplification of the five obesity-related genes from the Caco-2 cell line treated with a low concentration (a) and medium concentration (b) at day 42. PCR fragments were visualised on a 1% agarose gel stained with ethidium bromide. Lanes represent the following: M. KAPA Universal Ladder, 1. *WT1*, 2. *ATP10A*, 3. *CD44*, 4. *TNFRSF9,* and 5. *MEG3*.

4.3.2.5 PCR Analysis after 52 days of incubation

Amplification at day 52 for Caco-2 cells treated with a low concentration of sugar sweeteners resulted in clear bands for the *CD44*, *WT1*, *ATP10A*, and *TNFRSF9*. The *MEG3* appeared to have a band and a smear (Figure4.16). The molecular weight for the *WT1* was *390* bp, *ATP10A* was 399 bp, *TNFSRF9* was 390 bp, and *MEG3*was approximately 389 bp.The *CD44* gene had a low molecular weight of approximately 234 bp. Amplification for Caco-2 cell line treated with a medium concentration of the sugar sweeteners displayed clear bands for the genes that were amplified (*CD44*, *ATP10A*, *TNFRSF9*), whereas the *WT1* had an unclear band and the *MEG3* gene had a smear.

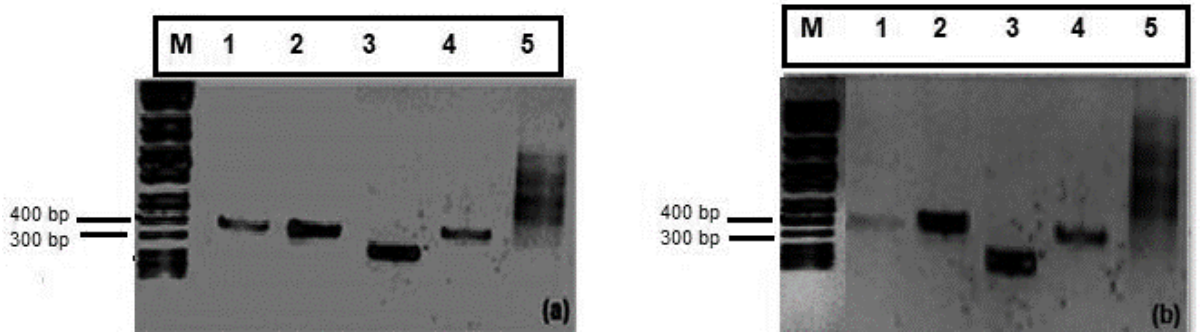

Figure 0.16: PCR amplification of the five obesity-related genes from the Caco-2 cell line treated with a low concentration (a) and medium concentration (b) at day 52. PCR fragments were visualised on a 1% agarose gel stained with ethidium bromide. Lanes represent the following: M. KAPA Universal Ladder, 1. *WT1*, 2. *ATP10A*, 3. *CD44*, 4. *TNFRSF9,* and 5. *MEG3*.

4.3.2.6 PCR Analysis after 62 days of incubation

Amplification at day 62 for Caco-2 cells treated with a low concentration of sugar sweeteners resulted in clear bands for the *WT1,CD44*, *ATP10A*, and *TNFRSF9*. The *MEG3* appeared to have assorted bands and unclear bands (Figure4.17). The molecular weight for the *WT1* was 390 bp, *ATP10A* was 399 bp, *TNFSRF9* was 390 bp, and *MEG3* genes were approximately 389 bp. *CD44* had a low molecular weight of approximately 234 bp. Amplification for Caco-2 cell line treated with a medium concentration of the sugar sweeteners displayed clear bands for the genes that were amplified (*CD44*, *ATP10A*, *TNFRSF9*), whereas the *WT1* and *CD44* genes had an unclear band and the *MEG3* gene had clear bands with a smear.

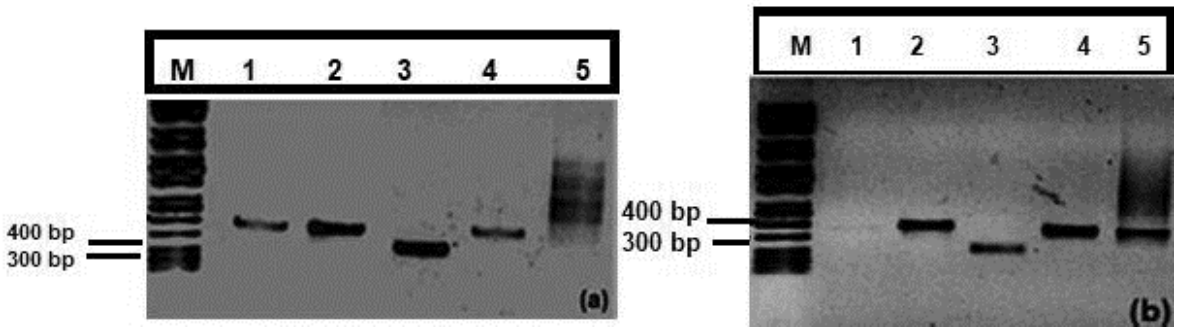

Figure 0.17: PCR amplification of the five obesity-related genes from the Caco-2 cell line treated with a low concentration (a) and medium concentration (b) at day 62. PCR fragments were visualised on a 1% agarose gel stained with ethidium bromide. Lanes represent the following: M. KAPA Universal Ladder, 1. *WT1*, 2. *ATP10A*, 3. *CD44*, 4. *TNFRSF9*, and 5. *MEG3*.

4.3.3 Methylation sensitive restriction enzymes digestion

MspI and HpaII were used for the digestion of methylated sites in the amplicon.The restriction enzymes HpaII and MspI both cleave the sequence CCGG, but the action of HpaII is inhibited if the internal cytosine is methylated. There was no cleavage on the amplicons, indicating that the restriction enzymes did not recognise any restriction sites on the amplicon DNA had no restriction site. The gel imaging (Figure 4.18) was the same for all concentration; thus, one picture used.

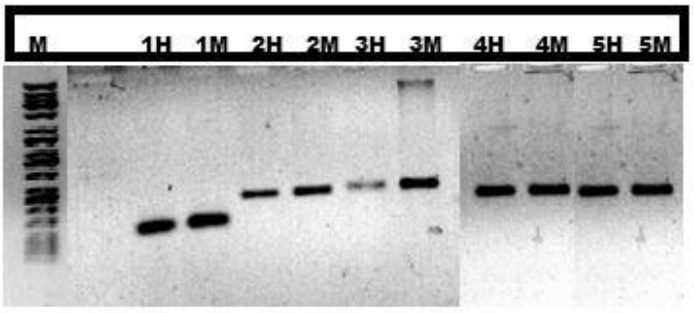

Figure 0.18: Restriction digestion from day 12- 62.L: Lane represents M: Universal ladder, 1c: control, 1H: *Cd44* with Hpall, 1M: *Cd44* with Mspl, 2H: *WT1* with Hpall, 2M: *WT1* with Mpl, 3H: *TNFRSF9* with Hpall, 3M: *TNFRSF9* with Mspl, 4H: *ATP10A* with Hpall, 4M: *ATP10A* with Mspl and 5H: *MEG5* with Hpall, 5M: *MEG5* with Mspl.

CHAPTER FIVE

DISCUSSION

5.1 Morphological characterisation of the caco-2 cell line

Carbohydrate digestion and absorption occurs mainly in the small intestine (SI) where sugar oligomers and disaccharides are broken down into monosaccharides by enzymes such as sucrase-isomaltase expressed on the apical membrane of the enterocyte (O'Brien and Corpe 2016).Thus, the use of the Caco-2 cells was necessary. Polarised Caco-2 monolayers represent a reliable correlation for studies on the absorption of drugs and other compounds such as sweeteners after oral intake in humans (Verhoeckx *et al*., 2015). Comparison of Caco-2 permeability coefficients with absorption data in humans has shown a high correlation, mainly if the compounds are transported by passive paracellular transport mechanisms (Verhoeckx et al. 2015). The cell population in the Caco-2 cell culture is characterised by subpopulations with different morphologies (Sambuy *et al.,* 2005). The Caco-2 cells were morphologically characterised after exposure to sweeteners. In Caco-2 cells, gastrointestinal intake is dependent on the integrity of the monolayer of cells and their tight junctions (Shappell, 2003) implying that if the morphology of the monolayer is changed, the intake of the sweeteners gets affected.

Figures 4.1 – 4.9 show that the higher the exposure of the sugar sweeteners, the more the cells differentiate as argued by Dailey (2014) that as in humans, the gene expression and morphology of the small intestine are altered during any high exposure to sucrose and obesity. These adaptive changes of the SI may be uncontrolled during high nutrient consumption and metabolic disease states. The small intestine primarily consists of enterocytes, which directly interact with dietary nutrients and transfer them into the circulatory system (Vereecke *et al.,* 2011). The rate of interaction between nutrients and enterocytes varies accordingto the eating habits of individuals.

5.1.1 Morphological Characterisation of the Caco-2 cell line for day one to eight exposures to sugars sweeteners of different concentrations

According to Jokhadar *et al.* (2009), Caco-2 Cell shapes were categorised into four shapes: type 1: rounded; type 2: cumulus shaped-semi-spread; type 3: fully spread; and type 4: elongated with some having long thin protrusions.This categorisation follows the chronological shape sequence. The shapes observed from day one to day eight of incubation was the rounded and the cumulus shaped (Figure 4.1-4.3). Caco-

2 cells in a suspension were observed to be spherical (Figure 4.1) and as they started to attach to wells, they flattened and spread as the incubation period progressed. Rounded cells at the start of treatment showed that the cells had not attached to the wells and at a later stage of the experiment they showed that the cells had detached from the wells and were floating in the medium.

The untreated cells (negative control) as shown in Figure 4.1a, 4.2a and 4.3a reached confluency at day eight of incubation. When polarised and confluent, the round cell spread layers forming an unceasing barrier between upper and lower compartments (apical/mucosal and basolateral/serosal)confirming the findings of Verhoeckx *et al.*(2015). The cells differentiated as they continued to grow polarised epithelial cell monolayer of round cells.The cells formed tight differential junctions holding the cells together forming an intact monolayer as displayed on the negative control (Figure 4.1 – 4.3a).

Hydrogen peroxide (positive control) was used because it is a known inducer of apoptosis. Apoptosis is a process whereby cells activate an intrinsic death program(Sidoti-De Fraisse *et al.,* 1998). At day one (Figure 4.1b), the cells were exhibiting signs of apoptosis characterised by the decrease in the number of cells and floating cells in the medium. Between day four and eight (Figure 4.2 – 4.3b), apoptosis occurred, characterised by floating cells. The length of the incubation period with H_2O_2 influenced the process of apoptosis. A longer incubation period led to all cells dying (Figure 4.3b). It is understood that apoptosis may be caused by oxidative stress, which may indicate DNA damage(Xiang *et al.,* 2016).

Compared to the negative control (which displays typical Caco-2 cell line), the cells treated with the low concentration of the sugar sweeteners at day one (Figure 4.1c-d),a disrupted monolayer was forming characterised by cells dissociating from each other due to the interactions with the low concentrations of the sugar sweeteners. A longer incubation with the low concentration of the sugar sweeteners resulted in numerous disrupted monolayers as seen in Figure 4.3c-d. A disrupted monolayer is as a result of mitochondrial *ATP*depletion (JanssenDuijghuijsen*et al.,* 2016). The*ATP*depletion may be due to the effect of the low concentration of the sugar sweeteners on the biological process.The cells treated with medium and high concentrations of sweeteners showed a different morphological change throughout

incubation, from day one to eight (Figures4.1 – 4.3e-h) compared to the low concentrations. The high and medium concentrations led to dissociation of cells at day eight. When the time of incubation (daysfour and eight) was increased, loss of cell density occurred due to the decrease in cell viability. Various reasons caused the decrease,one of which could be a reduction in metabolic activity as shown in Figures. 4.1f, 4.2f, and 4.3f.

There was variability in the cell morphologies,which was dependent on the sweetener concentration and time of incubation (Figures 4.1 – 4.3). Generally, Caco-2 cells were mostly used not as individual cells, but as a confluent monolayer on a cell culture confirming studies by Hidalgo *et al.* (1989)as seen in Figure.4.1 – 4.3a. The gradual uptake of the sweeteners at different concentrations changed the cell morphology due to the toxicity caused by the different concentrations. The above findings are supported by Jokhadar *et al.* (2009), who observed that Caco-2 cells' growth depended on environmental conditions.

5.1.2 Morphological Characterisation of the Caco-2 cell line for day 12 to 62 of exposure to different concentration of sugar sweeteners

Further morphological changes of the Caco-2 cell lines were influenced by the type of concentration and exposure duration.Cells treated wih 50% hydrogen peroxide from day 12 -62 showed cell death (Figure 4.4 – 4.9a). The length of the incubation period with H_2O_2 influenced the process of apoptosis. The cells treated with only fresh DMEM media displayed morphology of a typical Caco-2 cell (Figure 4.4 – 4.9b). As the incubation time increased, the cells became confluent and the cells' density increased. This resulted in cell death due to space limitation (Figures 4.7 – 4.9b).

The dead cells were characterised by the formation of a large cellular mass, dense irregular cellular debris floatingin the medium(Figure 4.5d,4.6c,4.7b-c,4.8c and 4.9b-c). Low concentrations displayed moderate toxicity on the cells. Some cells from the low concentration showed the morphology of typical Caco-2 cells (Figure 4.4b), but as the duration of the exposure progressed, the morphology changed in Figure 4.6 – 4.9b. The cells changed from round cells to an elongated shape with tapering ends. Elongated and tapering-ended cells later detached and diedThis was characterised by cells dissociating from each other and clumps of dark cells. Cells treated with medium

concentration (Figure4.4 – 4.9d) started showing a morphological change from day 12. The cells changed into variable shapes with tapering ends. Caco-2 cells, shortly before cell division, transformed into elongated shaped cells confirming studies and results obtained by Jokhadar *et al*. (2009).

At day 42, the cells appeared vacuolated (Figure 4.7d), and it was observed through to day 62 (Figure 4.9c-d).The cells had dissociated from each other. According to Shubin *et al.* (2016), cytoplasmic vacuolisation is a well-known morphological phenomenon observed in mammalian cells after exposure to bacterial or viral pathogens as well as to various natural and artificial low-molecular-weight compounds.In this study,the sugar sweeteners used were the compounds that triggered vacuolisation. Vacuolisation often accompanies cell death. Cell shrinkage was also observed. At a cellular level*, in vitro* toxicity can manifest in several ways, including, diminished cellular adhesion, dramatic morphological changes, a decrease in replication rate, and a reduction in overall viability as the data depicted. Over confluency was expected at the later stage of the study due to cell proliferation in the culture (Figure 4.7 – 4.9b).

Verhoeckx *et al.* (2015) stated that the Caco-2 cell line is heterogeneous and contains cells with slightly different properties. With this study, it can be deduced that the heterogeneity is dependent on the type of stage the Caco-2 cells have differentiated into and the type of experimental materials used on them.Cell adhesion to surfaces or their connection to other cells was dependent on their progress through the cell cycle, e.g., the passage of cells from interphase to mitosis and from mitosis to interphase is commonly marked by changes in cell adhesion as argued by Pugacheva *et al.* (2006). These changes in cell shape in this study are displayed in Figure 4.4 – 4.9. Cell shape changes are related to cellular processes such as growth, migration, differentiation, and apoptosis, as observed in several studies (Boudreau and Bissell, 1998; Schwartz and Ginsberg, 2002).

5.2 Cytotoxicity studies

5.2.1 Percentage viability of Caco-2 cells using Alamar blue

Alamar blue assay was chosen as one of the methods for evaluating the cytotoxicity of the sweeteners. Alamar blue assesses the metabolic activity of cells, which can be linked to cell viability because of the notion that dead cells are not expected to be metabolically active. Therefore, metabolic activity was used to assess the toxicity of the sugar sweeteners at varying concentrations over time. The metabolic activity of the treated Caco-2 cells was significantly influenced by the concentration of the sugar sweeteners and the duration of incubation (Figure 4.9). The period of exposure of eight days decreased cell viability (Figure 4.9). According to Alvarez and Shoichet. (2005), the toxicity of a substance is divided into four classes: Class 1 (high toxicity) with cell viability values between 0 to 25%; class 2 (moderate toxicity) with cell viability values between 26 to 50%; class 3 (low toxicity) with cell viability values between 51 to 75% and lastly class 4 (non-toxic) with percentage viability values higher than 75%.

Caco-2 cells treated with low sugar sweetener concentration had the highest viability across all the incubation periods indicating that the concentration had low- moderate toxicity to the cells, thus a steady decrease in cell viability (Figure4.9). The results showed that an increase in the concentration of the sugar sweeteners yielded an increase in toxicity to the cells; an increase in incubation time reduced cell viability. The cells only treated in fresh medium (negative control) maintained 100% cell viability (Figure 4.9). The positive control was highly toxic to the cells (Figure4.9), showing that hydrogen peroxide has a cytotoxic effect. Hydrogen peroxide significantly increases free radicals in the cells, resulting in critical DNA damage and oxidative stress as shown by Cai*et al*. (2013). H_2O_2-induced oxidative stress also increases cell apoptosis,a result that agrees with the findings of Jin *et al*. (2008). Apart from the dosage of the sugar sweeteners, Riss *et al.* (2004) stated that two other factors play a significant role in the toxicology of an entity; the duration of exposure to a compound and the compound's mechanism of toxicity.

The results showed that there was a reduction in metabolic activity across all days of incubation (Figure4.9). A reduction in metabolic activity in the mitochondrial functions indicated a potential loss in cell viability. The specific cellular pathways or particular

organelles that were affected by the presence of a different concentration of sugar sweetener inside the cell are unknown.However, the study showedthat exposure to sugar sweeteners resulted in cell death. Cell death can be induced either through apoptosis or necrosis (Pollard and Earnshaw, 2007). While apoptosis is the pathway for programmed cell death, activated through an intracellular program, necrosis is an accidental death resulting from a structural or chemical trauma, killing cells outright (Pollard and Earnshaw, 2007). Necrosis culminates in the spilling of the cytoplasmic contents into the surroundings due to increased membrane permeability, leading to undesired responses such as inflammation (*ibid.*). This loss of membrane integrity is not usually present in apoptosis.

5.2.2 LDH cytotoxicity assay

As a measure of cell health, LDH assay conducted provided information about the membrane integrity when cells were treated with the different concentrations of the sugar sweeteners. The LDH results showed that cell treatment with a low concentration (Figure4.10) across the different incubation periods had a gradual increase in LDH leakage, indicating that the low concentration had a low to moderate toxicity to the Caco-2 cells, hence the gradual release of LDH. The time factor worked hand in hand with the level of sweetener concentration to impact LDH leakage. Even if the duration of treatment were prolonged, low exposure triggered a gradual increase of LDH leakage. LDH from above 50% indicated that the treatment agent had a significant cytotoxicity effect in the cells**.**

Cells treated with the medium and high sweetener concentrations displayed a similar cytotoxicity trend with regards to the LDH leakage. At the most prolonged incubation period (eight days), LDH leakage increased abruptly for the medium and high concentrations indicating that these concentrations were toxic to the cells (Figure 4.10). With prolonged exposure, the leakage worsened, and the cells were damaged. The cells treated with high and medium sweetener concentrations showed that the more damaged a cell membrane was, the more leakage of LDH occurred. Cells treated with a high concentration of the sugar sweeteners had 70.25% in membrane rupture, showing that cell death occurred (Figure 4.10). Itwas also deduced that the LDH leakage increased within fewer days as the concentration of sweeteners increased.

Untreated cells showed that the LDH leakage was not significant. Throughout the different concentrations (Figure 4.10) and different incubation periods, the LDH leakage was below 20%, thus the conclusion that the leakage was not substantial.

The more damaged a cell membrane was, the more LDH leakage occurred. The rupture of the membrane occurred when Caco-2 cells were exposed to a high concentration of sugar sweeteners over time. The loss of intracellular LDH and its release into the culture medium indicated irreversible cell death due to cell membranes rupturingconfirming what Fotakis and Timbrell (2006) found in their study. Thus, the LDH is an assay that assesses the cytotoxicity effect rather than the cell viability whereas Alamar blue focuses on the cell viability.

In their study(Zhan *et al.,* 1997have shown that LDH leakage can be an indicator of either the necrosis or apoptosis cell death pathways. The secondary necrosis pathway explains this possible loss of membrane permeability in the apoptotic process (*ibid.*). Apoptotic cell bodies incur extracellular membrane rupture in the late stages of apoptosis (Majno and Joris, 1995). In normal conditions, phagocytes are responsible for the degradation of apoptotic cell bodies. Secondary necrosis occurs when no phagocytes intervene in the degradation step of apoptosis, proceeding to a necrotic phase in which cell bodies disintegrate and release the intracellular components into their surroundings as shown by Silva (2010).

Secondary necrosis is the usual outcome for apoptosis in *in vitro* experiments such as the one conducted in this study, as phagocytes are usually absent. In this study, there were no phagocytes present, an indication that the type of cell death that occurred was secondary necrosis. The absence of phagocytes would leadto a possible underestimation of apoptosis unless specific parameters were put in place (Gomez-Lenchon *et al.*, 2002). LDH leakage was a reliable measure of cell death and apoptosis if used in conjunction with other markers (Zhan *et al*., 1999). The LDH leakage results correlate with the finding of the Alamar blue assay. The findings of this study gave support to the understanding that different concentrations of sweeteners can have a significant effect on the viability and metabolic activity of the cells.

5.2.3Statistical analyses using ANOVA for the cytotoxic effect of the different concentrations of sugar sweeteners on the Caco-2 cell line

The ANOVA showedthat there was a significant difference to means relative to the untreated cells. The p-value less than 0.05, showed that the results were statistically significant (Sawyer, 2009). The low p-value with regards to the concentration suggested that it had the most significant contribution to the percentage viability of the cell (Table 4.6 and 4.7). The study also showed that the number of days of incubation ($p \leq 0.05$) affected cell viability significantly. The f critical values for the concentration and the number of days (Table 4.6 and 4.7) were less than the F value, and the p-value was less than 0,05, this resulted in the null hypothesis being rejected. This meant that the different levels of independent variables had a differential effect on the dependent variable. It can be interpreted that there was an effect of the different concentrations of sugar sweeteners over incubation time on the percentage cell viability of the Caco-2 cell line.

5.3 DNA methylationstudies

5.3.1 Bisulfite PCR

No amplification from the Bisulfite PCR was obtained; this process was done many times without results. New primers were designed, and the primer manufacturer was changed. Some of the problems inherent in bisulfite conversion were the poorly controlled efficiency of modification, which was incomplete due to incomplete denaturation or partial renaturation of DNA during treatment with similar outcomes being observedby Rein *et al.*,(1997). Troubleshooting techniques such as checking the DNA quality, warming the elution buffer to 70°C before elution to maximise the recovery of the DNA, increasing the concentration of $MgCl_2$ were performed. Performance evaluation was also done whereby bisulfite DNA was amplified with the primer pairs for bisulfite converted DNA. There were no amplicons compared to literature; if there was a complete DNA conversion, the bisulfite amplification was to yield bands as argued by New England Biolabs (2016). Whereas when bisulfite-treated DNA amplified primer pairs of unconverted DNAs, there were bands, and in literature, there should be no bands.

5.3.2 PCR analysis

There was no amplification observed for the bisulfite converted DNA. The amplification of the CpG islands was implemented as a contingency plan. The five obesity-related genes (*CD44, WT1, ATP10A, TNFRSF9*, and *MEG3*) were chosen for analysis based on their proposed functions in obesity. The study focused on the CpG islands of the genes. The CpG islands in the Caco-2 cell lines were successfully amplified. All the PCR products for day 12 to 62 (Figure 4.12 – 4.17) were similar. The expected base pairs for *WT1* gene was 380, 399 bp for *ATP10A*, 389 bp for *TNFRSF9*, 234 bp for *CD44* and 389bp for the *MEG3* genes according to the primer design. The results obtained showed that the molecular sizes of the genes were similar to the expected sizes. The *WT1* genes in all PCR products amplified at approximately 389 bp; in some days, the PCR products amplified had no bright bands; this could have been due to the quality, the concentration of the DNA or not enough template DNA was added in the reaction.

The *ATP10A, TNFRSF9, WT1* PCR products had similar molecular sizes. The *CD44* gene had a lower molecular weight out of all five genes; it amplified at around 230 bp. The *MEG3* gene amplified at approximately 390 bp but had multiple unclear bands.This could be because the primers may have annealed to different sites on the CpG islands. Due to the multiple bands, the amplification of the *MEG* 3 gene was repeated by changing and modifying the PCR parameters. The following troubleshooting steps were performed; increasing the annealing temperature and shortening annealing time usinga Gradient PCR, gradually increasing the $MgCI_2$ concentration, adding PCR additive BSA to enhance the amplification process and diluting the primer. The bands remained multiple, unclear, and smeared even after the troubleshooting steps.

The amplification of the CpG islands did not guarantee that methylation occurred. Due to factors such as the CG sequence at these locations may not be methylated. Many studies have revealed that DNA methylation occurs at sites other than the CpG sequences (Fotakis and Timbrell, 2006). As has been indicated before, DNA methylation is the most understood epigenetic mark that primarily occurs on the cytosine upstream of guanine (dinucleotide CpG) and is catalysed by the DNA

methyltransferases (DNMTs). The methylation of cytosines in a CpG has been shown to be sensitive to environmental stimuli.

5.2.3 Methylation sensitive restriction enzymes digestion

The enzymes used are the isoschizomers HpaII and MspI, both of which recognise the sequence CCGG. Whereas MspI is blocked only by methylation of the outer cytosine, HpaII is blocked by methylation of either cytosine. Since in mammalian genomes, methylation occurs chiefly in CpG sites, HpaII is inhibited, and MspI is not (Goll and Bestor, 2005). The main drawback of the method is the dependence of the availability of recognition sequences that flank the sequences of interest. No digestion of PCR products was observed (Figure4.18) except on the 3M. This might be due to the proximity of the recognition site to the end of the DNA fragment (Thermofischer Scientific 2020). Some restriction enzymes entail additional flanking bases for efficient DNA binding and cleavage. The data showed (Figure4.18) that the methylation-sensitive enzymes did not digest the amplicons, thus the conclusion is that there is no methylation.

CHAPTER 6

CONCLUSION

AND

RECOMMENDATIONS

6.1 Conclusion

Obesity is a chronic disorder on the rise globally, and wrong lifestyle choices may aggravate it. To study the transmission of obesity and obtaining solutions for its management, other factors such as epigenetics must be explored. Summary findings and conclusions per objectives and are provided in the following points**:**

1. **To stimulate the Caco-2 cells with varying concentrations of sugar sweeteners and assessment of the morphological changes of the cells**.

The morphological characterisation revealed that the Caco-2 cell, when exposed to different concentrations of sugar sweeteners, displayed different shapes; at the beginning of the treatment the cells were spherical, and as the treatment progressed, the Caco-2 cells flattened and differentiated into monolayers. Cell death, characterised by floating clumps of cells, occurred upon long exposure of the cells to the different concentrations of the sugar sweeteners. Further morphological changes of the Caco-2 cell lines were influenced by the type of concentration and exposure duration.

2. **To evaluate the cytotoxicity of different concentrations of the sugar sweeteners on the Caco-2 cells using the Alamar blue and LDH assay.**

Cell viability due to the effects of the different concentration of the sugar sweeteners strongly depended on concentration and exposure times, as depicted in the Alamar blue assay results. An increase in sugar sweeteners concentration and exposure time translated to an increase in cytotoxicity. The results of this work showed that cytotoxic effects are indeed more pronounced as time advances even beyond four days when treated with the high concentrations of the sugar sweeteners. The study showed that a low concentration of sugar sweeteners (fructose: glucose) used in beverages had low toxicity to the Caco-2 cell line and prolonged exposure of the low concentration might have an adverse effect on the cells. A medium concentration of the sugar sweeteners used in beverages had medium toxicity to Caco-2 cells, prolonging the exposure may lead to cell death. A high concentration was shown to be toxic to the cell. These findings suggested that control of dietary glucose: fructose intake is an important strategy to combat the development of obesity and type-2 diabetes.

3. **To obtain genomic DNA from the treated Caco-2 cells and carry out bisulfite conversion.**

The genomic DNA extracted from the sweeteners stimulated Caco-2 cell line was of good quality, purity, and the DNA concentration was sufficient for downstream applications. Bisulfite conversion of the genomic DNA was not successful.The digestion of the amplicons with two methylation-sensitive enzymes (Hpall and Mspl) was not successful.

4. **To amplify the *WT1*, *MEG3*, *TNFRSF9*, *ATP10A*, and *CD44* obesity genes and ascertain their degree of methylation.**

DNA methylation findings could not be established because the amplicons were not amplified by the bisulfite converted primers. The amplicons were also not digested by the methylation-sensitive enzymes used. As a contingency plan, regular PCR was performed to amplify the CpG islands of the five differential obesity-related genes, and there was amplification for all the five genes.

6.2 Recommendations

It should be highlighted that there are some limitations in the study that are inherent to the experimental design for this work. While the cell line used is an appropriate model for cytotoxicity studies *in vitro*, it may not entirely represent the response observed in other cellular tissues with different function and metabolic activity. Caco-2 cells do not reflect the general human status, for a functional study and trial, the test should be done on an actual human being. DNA methylation results from a human research study could lead to obtaining a biomarker for the generation of drugs for obesity. This would yield good results and an accurate interpretation of how DNA methylation influences obesity. *In vivo* tests would yield good results.

Alamar blue assay relies on an enzymatic transformation that may be modulated and altered by test compounds that might result in a misinterpretation of results. The endpoint of the assay might yield false positives and negatives due to the mechanism of toxicity of the test compounds.Therefore, caution with the interpretation of results should be exercised. Epigenetics can offer hope for disease research studies. DNA methylation findings could not be established as it is known that epigenetic effects are

gradual.A longer and gradual incubation period of seven months or a year might have effected DNA methylation.The percentage viability assay results of this study gave a good indication of the toxicity of the sugar sweeteners used in beverages. Still, the toxicological effect of the different sugar concentrations in humans was not established. This indicates that there is a new aspect of research to be explored that can concentrate *in vivo* cytogenetic studies in humans.

The proliferative action of Caco-2 cells, when treated with different concentrations of sweeteners, could be explored as potential research. Another related area of research is the assessment of the cytokines released in an obese individual.There is a correlation that in an obese individual, the number of hormones, cytokines, pro-inflammatory substances, and other substances which are involved in the development of insulin resistance are increased.

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