The effects of artificial and natural sweeteners

on various physiological systems

By

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Abstract

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Key words: Artificial sweeteners, natural sweeteners, sugar cane molasses, immune system, male reproductive system, female reproductive system

This study aimed to investigate the effects of commercially available natural (sugar cane molasses, white sugar and brown sugar) and artificial (Canderel™, Equal™, Natreen™, Sweetex™, Splenda™ and Swheet™) sweeteners on various physiological systems. The artificial sweeteners tested in this study may be categorised into their respective groups based on their primary ingredient. The brands Canderel™ and Equal™ contain aspartame, Natreen™ and Sweetex™ consist of saccharin and Splenda™ and Swheet™ are composed of sucralose. The inclusion of artificial or natural sweeteners in the human diet has been continually debated and their implication in the development of certain diseases has raised concern regarding their safe use. Therefore, it is necessary that these food products be subjected to a battery of tests to determine adverse effects on human health.

Firstly, we investigated the effect of the popular sweetener, sugar cane molasses on the immune system. Whole blood cultures were used to assess the impact of molasses on cytokines regulating specific immune pathways. Lactate dehydrogenase activity was used to determine potential cytotoxicity of sugar cane molasses. Results showed

that exposure of molasses to whole blood cultures caused no cell death. However, molasses stimulated interleukin-6 and interleukin-10 secretion, indicating effects on inflammation and humoral immunity respectively. The enhanced humoral response produced by molasses may be associated with increased antibody production that acts in defense against extracellular pathogens. Conversely, high IL-6 levels may be associated with the development of hypersensitivity reactions. Based on this outcome, we aimed to further investigate the comparative effect of molasses to other natural and artificial sweeteners using a similar model system. Results of this study showed that all artificial sweeteners had an inhibitory effect on the inflammatory response, while molasses stimulated the inflammatory process *in vitro*. The artificial, sucralose-containing sweeteners inhibited IL-10 under stimulatory conditions. Thus, exposure to sucralose induces a reduced humoral response that may be associated with adverse effects on the immune system.
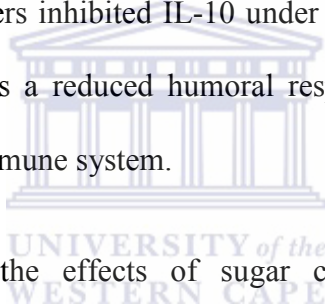
Secondly, we investigated the effects of sugar cane molasses on the male reproductive system. The effect of molasses on steroidogenesis was monitored using testicular cell cultures. Testes cultures were either stimulated or not stimulated with luteinizing hormone and exposed to molasses samples. The endpoints namely, lactate dehydrogenase activity, testosterone and estradiol synthesis were measured. Results show that molasses causes no cell death and has no impact on estradiol synthesis. However, molasses does exhibit a stimulatory effect on testosterone biosynthesis *in vitro*. Based on this outcome, we further investigated the comparative effect of molasses to various natural and artificial sweeteners. Results of these experiments indicate that in comparison to all tested sweeteners, molasses significantly enhanced testosterone secretion under stimulatory conditions. Perhaps

the supplementation of molasses in diets of males suffering with low testosterone levels may be of beneficial use.

The third objective of this study was to investigate the effects of natural and artificial sweeteners on the female reproductive system. Injury to the female reproductive tract was evaluated using frog, ovarian cell cultures. The biomarkers, testosterone and estradiol synthesis were measured to determine the impact of sweeteners on ovarian steroidogenesis. Cultured, oocyte fragments (*Xenopus laevis)* were exposed to either natural or artificial sweeteners under stimulated or unstimulated conditions. Results showed that the artificial sweetener, Natreen™ elevated testosterone levels while the natural sweetener, molasses enhanced estradiol levels. These results were supported by a correlating decrease on the E_2/T ratio by Natreen™ and an increase in the E_2/T ratio by molasses. Exposure to the aspartame and sucralose branded sweeteners reduced estradiol synthesis and this was confirmed by the decrease observed in the E_2/T ratio. These results suggest that certain sweeteners have potential androgenic, estrogenic or anti-estrogenic characteristics that may be associated with either harmful or beneficial effects on the female reproductive system.

Sugar cane molasses demonstrated a significant effect on all investigated physiological systems *in vitro.* In order to validate these results, our final objective was to determine the impact of molasses on physiological systems using *in vivo* and *in vitro* methods. For this study, Balb/C, male mice were given an oral dosage of molasses for the exposure period of two months. Animals were allocated into either a molasses-treated or a control group. Parameters such as body weight, physiological changes and molasses intake were measured. Collected blood samples were assayed

for potential toxicity using plasma biomarkers and liver enzyme activity. The effect of molasses on the immune system was investigated by monitoring antibody titre levels in immunised treated and untreated animals. Testes harvested from treated and untreated groups were cultured and assayed for testosterone synthesis. This was used to determine the impact of molasses on the process of testicular steroidogenesis. Results showed that molasses-treated groups consumed a significant amount of fluid and displayed symptoms of loose faeces. No significant change on body weight was observed in both treated and untreated groups. Plasma biomarkers and liver enzymes remained unaffected in molasses-exposed animals. However, a decrease in levels of IgG anti-antigen in treated groups indicated that molasses suppresses the humoral immune response. This may be associated with an increased risk to infection with extracellular pathogens. Molasses-treated animals significantly elevated levels of testosterone production *in vitro*. This result was consistent with previous findings and supports the use of molasses as a supplement to augment testosterone synthesis.
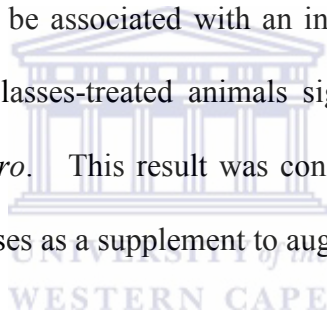
Declaration

I declare that "The effects of artificial and natural sweeteners on various physiological systems" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by references.

Full name: Farzana Rahiman   Date: 19 May 2011

Signed:

Dedication

To my Heavenly Father, Lord and Saviour, Jesus Christ. I am grateful for His unending favour and supernatural blessing over my studies for the past nine years. I thank you Lord for granting me the strength, discipline and opportunity to complete my PhD at the University of the Western Cape. To my amazing grandmother, Angel Gopaulsamy, who has been a strong pillar in my life. I thank you for your love and support throughout the years.
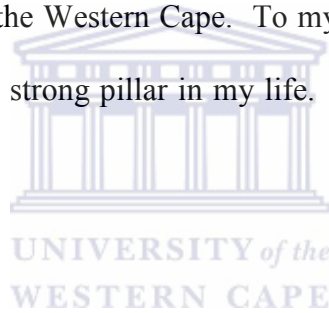
Jeremiah 29:11

'For I know the plans I have for you,' declares the LORD, 'plans to prosper you and not to harm you, plans to give you hope and a future.'

Acknowledgements

Contents

List of abbreviations

ADCC   - Antibody dependent cellular cytotoxicity

AIDS   - Acquired immunodeficiency syndrome

ALP   - Alkaline phosphatase

ALT   - Alanine aminotransferase

ANOVA   - Analysis of variance

AP   - Alkylphenols

APCs   - Antigen presenting cells

APEOs   - Alkylphenol polyethoxylates

AST   - Aspartate aminotransferase

B-HSD   - Beta-hydroxysteroid dehydrogenase

BPA   - Bisphenol A

BSA   - Bovine serum albumin

BW   - Body weight

CFU-GM   - Colony forming unit-granulocyte macrophage

Con A   - Concavalin A

CO_2 - Carbon dioxide

CYP 19   - Enzyme aromatase

CsA   - Cyclosporine A

DDE   - 1, 1-dichloro-2, 2-bis (*p*-chlorophenyl) ethylene

DDT   - Dichlorodiphenyltrichloroethane

DEHP   - Di-(2-ethylhexyl) phthalate

DES   - Diethylstilbestrol

DTH   - Delayed hypersensitivity

DMSO   - Dimethyl sulfoxide

E_2	- Estradiol
EDCs	- Endocrine disruptors
ELISA	- Enzyme linked immunosorbent assay
ERT	- Estrogen replacement therapy
FSH	- Follicle stimulating hormone
g	- grams
g/kg	- gram per kilogram
g/L	- grams per liter
Hct	- Hematocrit
HCL	- Hydrochloric acid
HIV	- Human immunodeficiency virus
HRP	- Avidin horseradish peroxidase
H_2SO_4	- Sulphuric acid
IgM	- Class M immunoglubulin
IgG	- Class G immunoglobulin
IgD	- Class D immunoglobulin
IgE	- Class E immunoglobulin
i.p.	- Intraperitoneal
IFNs	- Interferons
IFN-γ	- Interferon-gamma
IL-4	- Interleukin-4
IL-6	- Interleukin-6
IL-10	- Interleukin-10
IL-12	- Interleukin-12
IU/L	- International units per liter

kg	- Kilograms
LDH	- Lactate dehydrogenase
LH	- Luteinising hormone
LPS	- Lipopolysaccharide
MALT	- Mucosa associated lymphoid yissue
MCF-7	- Breast cancer cell line
MHC II molecules	- Major histocompatability complex II molecules
mg/kg	- Milligrams per kilogram
mg/ml	- Milligrams per millilitre
MS222	- 3-aminobenzoic acid ethyl ester
mU/ml	- Milliunits per millilitre
n	- Sample size
NAOH	- Sodium hydroxide
NIH 3T3	- Mouse embryonic fibroblast cell line
ng/mg	- Nanograms per milligram
ng/ml	- Nanograms per milliliter
nm	- Nanometers
NOEL	- No observable effect level
NPs	- Nonylphenols
NPEOs	- Nonylphenol polyethoxylates
PCBs	- Polychlorinated biphenyls
°C	- Degrees centigrade
OD	- Absorbance
OECD	- Organisation of Economic Co-operation for Development
PCDDs	- Polychlorinated dibenzo-p-dioxin

PCDFs	- Polychlorinated dibenzofurans
PCOS	- Polycystic ovarian syndrome
pg/ml	- picograms per milliliter
PHA	- Phytohemagglutinin
R^2	- Coefficient of correlation
rpm	- revolutions per minute
RPMI	- Roswell Park Memorial Institute
SEM	- Standard error of the mean
SLE	- Systemic lupus erythematosus
SRBCs	- Sheep red blood cells
T	- Testosterone
Th cells	- T helper cells
Tc cells	- T cytotoxic cells
TCDF	- Tetraclorodibenzofuran
TCDD	- Tetrachlor-dibenzodioxin
TMB	- Tetramethylbenzidine solution
TNFa	- Tumor necrosis factor alpha
TP	- Total protein
µg/ml	- Micrograms per milliliter
µg/µl	- Micrograms per microliter
µl/well	- Microliters per well
WBCs	- Whole blood cultures
w/v	- Weight per volume
w/w	- Weight per weight
x g	- Gravitational force

List of figures

List of tables

Chapter 1

Introduction: The effects of natural and artificial sweeteners on various physiological systems

Advancement in the food industry has lead to the development of a variety of sweeteners made available to the consumer. Sweeteners provide an enjoyable taste that is accompanied with energy (nutritive) or without energy (non-nutrtitive) (American Dietetic Association (ADA), 2004). Sugar is a worldwide, nutritive sweetener that is derived from either sugarcane or sugar beet. Sugar has become a vital ingredient in the human diet and is used for its ability to enhance palatability and preserve various food products (Nayaka et al., 2008). Reports suggest that elevated sugar consumption may result in an increase in body weight and chronic diseases related to obesity and dental caries. Cancer, heart problems and diabetes are some of the health conditions associated with excessive weight gain (Sardesai and Walshan, 1991).

Artificial sweeteners namely, aspartame, saccharin, cyclamate and acesulfame-K satisfies the consumer's need for sweet taste but without the added energy intake (Rogers and Blundell, 1989; Greenly, 2003). These low-calorie sweeteners are advertised by the food sector for their proposed health benefits such as reduced risk factors for obesity and associated diseases, and management of disorders such as diabetes. Conversely, other reports imply that the use of artificial sweeteners may induce severe health problems such as seizures, allergic reactions, mood swings, difficulty in breathing, headaches and cancer (Zygler et al., 2009).

Due to the contradictory reports regarding safety, benefits and risks of sweeteners, it is necessary to subject individual sweeteners to scientific tests to elucidate their safety. This knowlegde will provide us with information as to the safe use of a food product and enable us to predict any adverse effects on human health.

1.1 Subject objective

The main aim of this study was to investigate the effects of natural and artificial sweeteners on various physiological systems. Both *in vitro* and *in vivo* model systems were used to monitor the potential adverse effects of sweeteners on human health. Firstly, we investigated the effects of the natural sweetener, sugar cane molasses on the immune system and the male reproductive system using *in vitro* assays. Based on the outcome of this objective, we aimed to further investigate the comparative effect of sugar cane molasses to other natural and artificial sweeteners. The *in vitro* effects of sweeteners on physiological systems such as the immune and reproductive systems were investigated. Sugar cane molasses exhibited significant effects and as a result we further analyzed the impact of molasses in the rodent model using a combination of *in vivo* and *in vitro* tests.
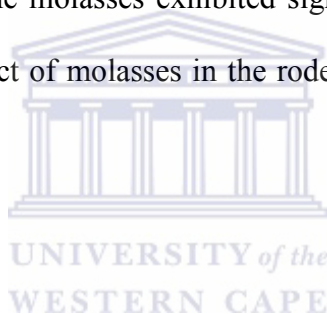
1.2 Study rationale

Natural and artificial sweeteners form an integral part of the food industry and the human diet. The use of these food items still remains a controversial topic. In light of the development for better or improved sweeteners, it is imperative that we screen such food products to determine potential adverse effects on consumer health.

1.3 Natural and artificial sweeteners

The human population inherently prefers sweet taste (Grotz and Munro, 2009). Research has shown that newborn babies with developed taste buds have a natural fondness towards sweetened solutions (Sardesai and Waldshan, 1991). Sweetness can act as a sensory indicator for energy, required for both metabolic and physical function. Sweet taste is a pleasurable sensation that has been used to camouflage distasteful products, thereby increasing the palatability of certain foods and medications (ADA, 2004).

The early *Homo sapiens* fulfilled their desire for sweetness through the intake of natural, sweet-tasting foods such as vegetables and fruits. Later, the expansion and development of the food industry lead to wide-scale production of refined sugar processed from either sugar cane or sugar beet. Refined sugar became readily available to consumers at affordable prices and this resulted in an increase in the use of sugar worldwide (Sardesai and Waldshan, 1991). The demand for sugar use has lead to the manufacture of a variety of sweeteners that are currently available on the market, today. These sweeteners may be categorised as either nutritive sweeteners that provide energy (calories) or non-nutritive sweeteners that provide intense sweetness but without energy (non-caloric) (Grenby, 1991, ADA, 2004).

1.4 Nutritive (natural) sweeteners

Nutritive sweeteners are composed of either monosaccharides or disaccharides that function as the body's primary source of fuel (Greenly, 2003). Sugars, often referred to as culinary sugars (used in food preparation) have emerged as a vital component of both our diet and culture (Sugar Researsch Advisory Service (SRAS), 2004; Nayaka et al., 2009). In the food industry, sugar is used to sweeten various drinks (tea, coffee and other beverages) and breakfast cereals. Powdered sugars, which contain cornstarch, are used as baking agents, frostings, icings and in uncooked candies. Sugar acts as a humectant by sustaining the water content of food products, thereby increasing its shelf life and also acts as a preservative by inhibiting the growth of bacteria, moulds and yeast (SRAS, 2004). Refined sugar is consumed largely by children in the form of ice creams, candy bars, biscuits and sweets and is also used extensively by the pharmaceutical sector (Wyse, 1986).
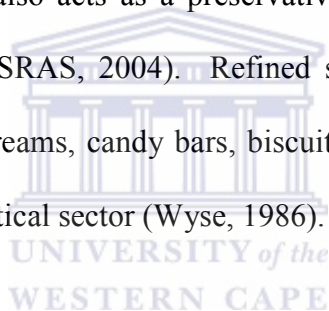
The sugar refinement process

Culinary sugars may be classified into various types based on the process of manufacture, size and nature of sugar crystals, as well as the palatability and colour of sugars. The most frequently used sugars are white refined (also referred to as blanco directo), brown and raw sugar (Nayaka et al., 2009). Sugarcane is a well-recognized agricultural crop that is primarily cultivated as a source of sugar (Daniels and Roach, 1987). Once harvested, sugar cane is transported to the sugar refinery where it is crushed and shredded. A process of extraction occurs, where the cane juice is removed and the by-product referred to as molasses remains. The cane juice is then treated with lime (calcium dioxide) to eliminate any impurities and carbon dioxide to remove the surplus lime. This is followed by a process of clarification, where the

resultant, concentrated syrup is crystallized in a screen-basket centrifuge. The purpose of centrifugation is to separate the majority of the syrup from the crystals. This process is repeated a few times. The clarified juice is then boiled and dried to produce raw sugar, which is sold in bulk to sugar refineries worldwide. Molasses is the residual, thick syrup left after sucrose has been extracted from the clarified sugar juice (Mackintosh, 2000).

Figure 1.1 The sugar cane plant growing in a field situated in Kwa-Zulu Natal, South Africa

Sugar refinement entails raw sugar being processed further. To remove the residual molasses retained by raw sugar, it is washed and dissolved in water syrup. A step of filtration then follows that functions to remove impurities and discolourants. Finally, to crystallize the sugar it is processed through steps of heating, washing and centrifugation. Granulated, white sugar is the end product of this process and can now be sold to the public. Granulated sugar can be categorized in accordance with its crystal size, falling under either 'extra fine,' 'fine,' 'super fine (powdered sugar)' and 'coarse' grades of sugar (Severn, 1987). During the late stages of sugar refinement, fine sugar crystals combine with large amounts of molasses to form brown sugar (Nayaka et al., 2009).

In India, certain companies produce raw sugar in the form of loaves also termed 'jaggery sugar'. Jaggery sugar, consisting of yellow to brown sugars, is manufactured by cooling down clarified cane juice to a solid crystalline form after reaching boiling point. This minimises the process of refinement and aids in maintaining mineral salts and phytochemicals. Ayurvedic practitioners propose jaggery sugar to be of beneficial use in the treatment of lung and throat infections (Nayaka et al., 2009). Evidence also suggests that jaggery sugar can be associated with the prevention of lung injury in rats exposed to compounds such as coal and silica (Sahu and Saxena, 1994). A study investigating the potential effects of jaggery and other sugars such as white, refined and brown sugar on NIH 3T3 fibroblasts and human erthrocytes reported that both jaggery and brown sugar displayed cytoprotective potential against hydrogen peroxide and tert-butyl hydroperoxide induced oxidative damage. The cytoprotective and antioxidant properties of the above-mentioned sugars may therefore support their role as sweeteners and therapeutics (Nayaka et al., 2009).
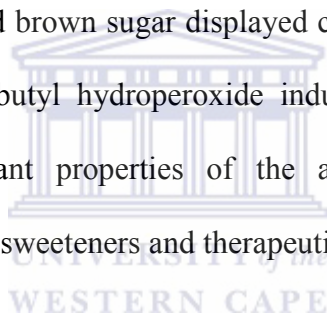
Sugar cane molasses

Sugar cane molasses is a heavy, viscous substance derived as a by-product of the sugar refinement process. Molasses has become an integral part of the human diet and is currently a popular ingredient, being used to sweeten hot drinks and alcoholic beverages. Anecdotal reports suggest that molasses may be used as a supplement in the human diet to improve conditions such as constipation, varicose veins, nerve damage, eczema, high blood pressure, dermatitis, anaemia, colds, coughs, ear aches, arthritis, ulcers, hair damage and bladder problems (Kirschmann, 2007; Reyed and El-Diwany, 2008; Crellin et al., 1990). In addition, anecdotal evidence proposes that the purest form of molasses may be used in the treatment of cancer. It has been

hypothesised that plantation workers on sugar cane fields, who frequently consume raw, brown sugar have a reduced risk to developing cancer. Sugar cane molasses contains fundamental amino acids and lineolic acids, which have been reported to have anti-tumor potential. Different minerals such as selenium, iron, magnesium, zinc, potassium and bulk of the vitamin B complex also constitute to the make-up of molasses, which may be effective in reducing the risk to different forms of cancers (Grandics, 2003).

The adverse effects of sugar

Sugar has been classified as a non-toxic substance that has had a long history of safe use (Daniels and Roach, 1987). There is a lack of support that directly relates sucrose intake with toxicity, however there are several reports that suggest sugar consumption may be related to various adverse consequences (Howard and Wylie-Rosett, 2002). The development of autoimmune and cardiovascular diseases, cancer, dental caries, gallstones, premature aging, appendicitis, depression, hyperactivity and various other metabolic disorders have been associated with an increased dietary intake of sugar (Howard and Wylie-Rosett, 2002; Appleton, 1996). In 1974, the term 'saccharine disease' was defined as all conditions associated with the consumption of refined carbohydrates such as sugar and flour (Cleave, 1974). It was proposed that the refinement of carbohydrates cause harmful effects that manifests in three major ways in the body. Firstly, the loss of fibre results in constipation with its consequential impact on the stomach (diverticular disease), colon (cancer) and lower venous system (varicose veins, haemorrhoids, deep venous thrombosis and varicocele). The removal of fibre in one's diet may also be connected to the development of dental caries (in combination with a high sugar diet) as well as periodontal disease. Secondly, over-

consumption of these carbohydrates result in diseases such as diabetes, coronary thrombosis, gall stones, obesity and primary *Escherichia coli* infection. Lastly, the loss of protein in an individual's diet may be associated with peptic ulceration (Cleave, 1974).

In the mid-19th century, sugar was perceived as a 'pure white and deadly' substance due to the attribution of several harmful effects to it (Heasman, 1989). Recent scientific evidence suggests that sugar is not in itself associated with chronic diseases such as diabetes, coronary heart disease, obesity and hyperactivity in children. A research study conducted in the United States showed that women consuming sucrose and carbohydrates did not have a greater risk of developing diabetes. However, results did reveal that diets containing a high glycaemic load correlated with increased consumption of refined grains and an elevated risk to the development of diabetes (Mardis, 2001). Mardis (2001) also states that when taking epidemiological studies in consideration it is evident that sugar is not the only factor that contributes to the progression of diabetes. Controlled studies have also shown that there is no significant relationship between the consumption of sugar and the hyperactive behaviour in children (Mardis, 2001).

Anecdotal reports suggest that molasses is beneficial in improving health conditions; however, contradicting evidence implies that molasses may be associated with adverse effects. In 1967, researchers discovered that maturing cattle fed large amounts of molasses developed a syndrome termed 'molasses toxicity' (Pate, 1983). This condition is characterised by symptoms that affect the coordination of the legs and head (Lora et al., 1977). Although 'molasses toxicity' was initially believed to be a result of a mineral deficiency, it was later recognised by Verdura and Zamora (1970)

as a condition caused by cerebrocortical necrosis or polioencephalomalacia. Another metabolic problem related to high levels of molasses included in the diets of cattle is loose faeces, which commonly leads to diarrohoea (Pate, 1983). Urea toxicity and bloat are metabolic disorders that occur in animals fed a diet containing molasses as either a supplement or forming majority of the feed. Animals exposed to molasses/urea mixtures consume approximately 300 g urea per day (for 500 kg animal) and seldom appear to develop symptoms of urea toxicity; as sugars present in molasses and ammonia from urea are rapidly taken up for microbial expansion. However, toxicity occurs when urea is not evenly disseminated or if problems occur in the formulation of the feed (Preston et al., 1986; Jarige and Beranger, 1992). Bloat is hypothesised to be initiated by overgrowth of microbes within the rumen of animals and this leads to over-production of mucilaginous secretions (Jarige and Beranger, 1992). These disorders may be correlated with poor health condition of animals that may incur great losses for farm owners.
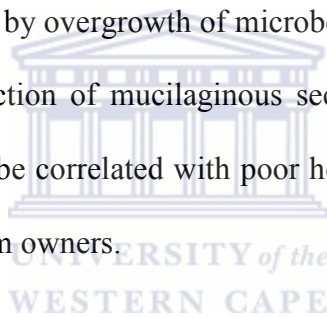
In the 1990's, beef and dairy farmers in South Africa observed a chronic syndrome in their livestock. Effects of this syndrome included reduced weaning weights, suppressed immune responses in already immunocompromised animals as well as an elevation in reproductive diseases. Farmers believed that the consumption of sugar cane molasses or molasses products were the primary cause for such effects. This syndrome was characteristic to that of an "endocrine disruptive syndrome" (Masgoret et al., 2009). Masgoret and colleagues (2009) investigated the endocrine disruptive potential of sugar cane molasses in cattle feed. Initially, four groups of molasses were investigated using well-established *in vitro* assays for inclusion in an *in vivo* study. Only two groups of molasses were chosen as treatments for the feeding trial. This study comprised of 4-6 week, Holstein bull calves that were categorised into either

treatment or control groups. Treatment groups consisted of calves fed rations comprising of the different batches of molasses while the control group was exposed to rations lacking in molasses. Although molasses displayed probable endocrine disruption *in vitro*, *in vivo* data showed that molasses had no endocrine disruptive effects in calves (Masgoret et al., 2009).

1.5 Non-nutritive (artificial) sweeteners

Non-nutritive or intense sweeteners provide the sensation of sweet taste without the calorie intake (Grenby, 1991). This enables consumers to reduce their calorie consumption and helps maintain body weight (Grotz and Munro, 2009). Artificial sweeteners are widely included as ingredients in beverages, food as well as in baking and medicinal products. Their beneficial effects have been advertised on a large scale by the food industry. Along with helping to control body weight, artificial sweeteners are also recognised for their ability to manage disorders such as diabetes and obesity. In addition, non-nutritive sweeteners are regarded as tooth-friendly products since they cannot be fermented by the microflora of dental plaque. Thus, artificial sweeteners may be associated with a lowered risk in the development of dental caries (Zygler et al., 2009).
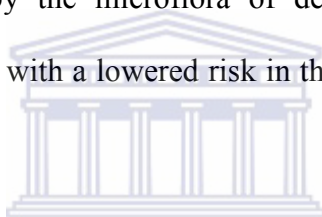
Although artificial sweeteners have been applauded by consumers for its beneficial effects, reports suggest that these sweeteners are linked to severe disorders such as respiratory problems, headaches, cancers, allergies, seizures and immune system dysfunction (Zygler et al., 2009). Therefore, it is necessary to investigate the potential toxicity of these compounds as well as determine concentrations as to which they can be safely used. There are currently various types of artificial sweeteners such as aspartame, acesulfame-K, neotame, cyclamate, sucralose and saccharin that are commercially available (ADA, 2004). This study aims to investigate the potential adverse effects of three of the previously mentioned sweeteners i.e. aspartame, saccharin and sucralose.

Aspartame

Aspartame was endorsed by regulatory authorities for consumer use over twenty years ago. Since then, this high-intensity sweetener has become a commonly used food additive in over six thousand products that are available worldwide (Butchko and Stargel, 2001; Fisher, 1989). In the food industry, aspartame has been branded under the labels such as 'Equal', 'Nutrasweet' and 'Canderel' and in its concentrated form is approximately 180 times sweeter than sucrose (Grenby, 1991). Aspartame is a dipeptide that is metabolised into three main constituents namely aspartic acid, phenylalanine and to a small extent methanol that are generally present in both the body and diet (Butchko and Stargel, 2001; Janssen and Van der Heijden, 1988).
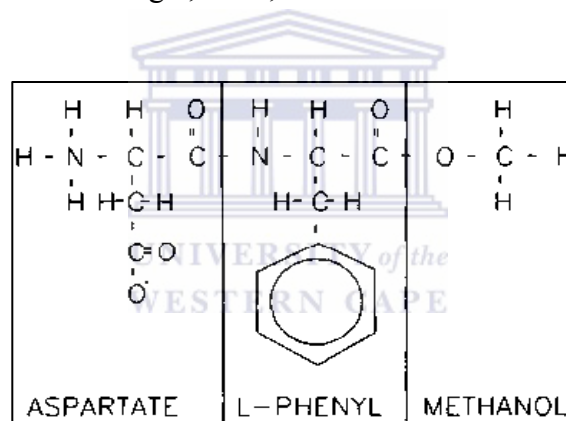
Figure 1.2 The chemical components of aspartame (Fisher, 1989)

It has been hypothesized that the consumption of aspartame may lead to various adverse effects such as seizures, headaches, allergies as well as impairment in behavioral and cognitive function (Butchko and Stargel, 2001). For this reason, aspartame has become the focus of various toxicity studies over the years (Janssen and Van der Heijden, 1988). Allergic responses to aspartame have been reported to manifest as skin reactions, edema of the tongue, lips and throat as well as difficulty in respiration. On the contrary, challenge studies have indicated that it was difficult to

find individuals who suffer from aspartame allergies and also report that their efforts were futile in replicating allergic responses under controlled, experimental conditions (ADA, 2004).

Research has shown that exposure of pregnant and lactating rats to large quantities of aspartame has an adverse impact on their offspring. High levels of aspartame intake increased body and brain weights, prolonged morphological and reflex growth and elevated mortality rates in offspring of exposed mothers. The amino acid, phenylalanine, which is a constituent of aspartame was believed to be the primary cause of these harmful effects (Holder and Yirmiya, 1989). Phenylalanine has also been implicated in affecting behavior and may be involved in inducing seizures by altering neurotransmitter levels (Sardesai and Waldshan, 1991).

A study investigating the effect of aspartame on the incidence of migraine headaches in migraineurs revealed that the occurrence of headaches was significantly amplified, however not in the length or intensity of headaches. These results were nevertheless criticised due to problems arising in statistical analysis of data that contributed to non-significant deductions. Conversely, a double-blinded, placebo study assessed individuals who claimed to have developed headaches as a result of aspartame intake and found that aspartame had a similar effect when compared to the placebo. Thus, it was concluded that this high-intensity sweetener was not a causal factor of headaches experienced by subjects (Butchko and Stargel, 2001).

Saccharin

In 1879, the chemist, Constantine Fahlberg fortuitously discovered saccharin and its role as an artificial sweetener grew rapidly in the food industry (Schiffmann and

Gaitlin, 1993). Saccharin is approximately three hundred times sweeter than sucrose and has been commercially marketed under the brand name 'Sweet 'N Low'. Saccharin is an important ingredient in various food and health products such as toothpaste, beverages and mouthwashes and is made easily accessible to international communities (Greenly, 2003).
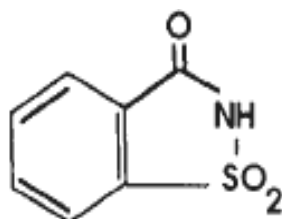
Figure 1.3 The chemical structure of saccharin (Sardesai and Waldshan, 1991)

The use of saccharin-containing sweeteners has stirred a lot of controversy amongst regulatory authorities worldwide (Grenby, 1991). In 1977, the FDA prohibited the public use of saccharin products because evidence suggested that this artificial sweetener acted as a carcinogen by playing a significant role in the development of bladder tumors in rats (ADA, 2004; Schiffmann and Gaitlin, 1993). In contrast, other research studies show that saccharin usage and the development of cancer cannot be correlated (Grenby, 1991). A lengthy study investigating the exposure of sodium saccharin (25 mg included in diet/kg every day for 5 days/week) to 20 monkeys demonstrated that none of these animals displayed any signs of urothelial proliferations or bladder cancer. However, this study was criticized by the scientific community for having a small sample size and for administering saccharin at a concentration that was not applicable to human consumption (Weihrauch and Diehl, 2004).

Luini et al. (1981) reported that the effects of saccharin on host defenses showed that the excretion of saccharin occurs without the synthesis of metbolites *in vitro*. Results also showed that exposure to saccharin, suppressed responses of rat lymphocytes to phytohaemagluttinin (PHA), but had no impact on macrophages or natural killer cells. An extension of this study aimed to determine whether the inhibitory effect of saccharin *in vitro*, displays any significance *in vivo*. Results of *in vivo* experiments revealed that rats fed elevated concentrations of saccharin showed decreased antibody synthesis against sheep red blood cells. As a result, the suppression of antibody production may be associated with an inhibitory and therefore adverse effect on the humoral immune system. On the other hand, no impact of saccharin on PHA blastogenesis was observed and this was inconsistent with *in vitro* data obtained for this study (Luini et al., 1981).
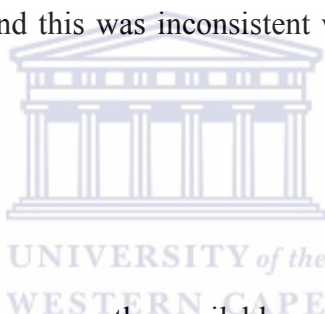
Sucralose

Sucralose is one of the more recently available, commercial sweeteners and is estimated to be six hundred times sweeter than sucrose. Sucralose is derived from sucrose by three hydroxyl groups replacing three chlorine atoms (Sardesai and Waldshan, 1991). This high-intensity sweetener is used as an additive in food products, beverages and medications. Sucralose proves to be very beneficial to producers as it is extremely stable at high temperatures, which is necessary to maintain the sweet taste following processes such as pasteurisation, cooking and baking (Grotz and Munro, 2009). Although sucralose tastes similar to sugar except for having an aftertaste, it does not contribute to dental decay (Greenly, 2003). In the body, sucralose is poorly metabolised, it fails to bioaccumulate and is eliminated unaffected in the faeces of mice, rats, dogs and humans. Since sucralose is not

identifiable as a sugar or carbohydrate within the body, it has no impact on glucose levels in diabetic patients (Greenly, 2003; Grice and Goldsmith, 2000).
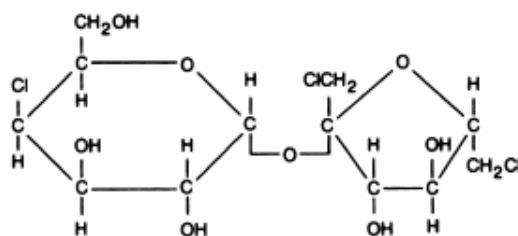
Figure 1.4 The chemical structure for sucralose (Grice and Goldsmith, 2000).

Sucralose has been intensely scrutinized and subjected to vigorous toxicity testing in order to determine its safe use (Grotz and Munro, 2009). In 1999, the FDA supported the use of sucralose as a high intensity sweetener. This was based on extensive research that included over 110 studies investigating the potential carcinogenic, reproductive and neurologic effects of sucralose in both humans and animals. Results showed that sucralose had no adverse effects on the human body and as a result, the FDA concluded that sucralose was safe for human consumption (ADA, 2004).

Goldsmith (2000) used acute and subchronic toxicity testing to determine the potential toxicity of sucralose. The acute oral administration of sucralose at concentrations of 10 and 16 g/kg revealed no toxic impact in both rats and mice. In a dietary study over a 4-8 week period, it was also demonstrated that no adverse effects were seen in rats exposed to doses up to 2.5 % of sucralose. However, the dietary treatment of 5 % sucralose to animals resulted in effects such as reduced food intake, body weight and food conversion efficiency. Organs such as the spleen and thymus were also observed to have decreased weights and modifications to their histopathological appearance (Goldsmith, 2000).

Over the years, research has produced contradicting evidence suggesting that both nutritive and non-nutritive sweeteners have potential adverse or beneficial effects on the human body. Although the use of most artificial and natural sweeteners have been approved by regulatory authorities such as the FDA, concern still remains regarding whether high levels of sweetener consumption affect optimal diet and health (ADA, 2004). It is therefore important that toxicity testing on these sweeteners be conducted to determine their safe use. This study aims to investigate the effects of frequently used nutritive (sugar cane molasses, brown sugar and white sugar) and non-nutritive (aspartame, saccharin and sucralose) sweeteners on various physiological systems of the body. Such systems include the immune system as well as the male and female reproductive systems.

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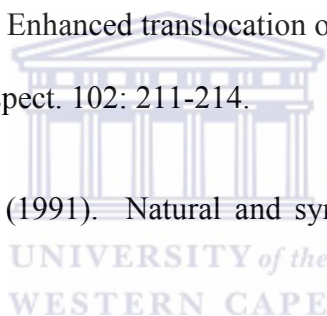
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Chapter 2

The effect of natural and artificial sweeteners on physiological systems

2.1 Introduction

Food products contain certain synthetic chemicals that function in increasing their flavour or shelf-life. However, synthetic chemicals such as pesticides or environmental compounds may also exist as contaminants in food items. Concern has been raised as to whether these compounds increase the risk to development of certain conditions such as cancer. Natural food additives of plant and fungal sources have also demonstrated their ability to induce harmful effects in both animals and humans (Abbott, 1992). Therefore, natural and artificial additives may have the potential to adversely affect vital organs or physiological systems. This study focuses on determining their effects on the immune system and the male and female reproductive systems.

2.2 The immune system

The immune system is a complex network of immune cells, tissues and organs that function together in defending the host from invading foreign agents such as fungi and parasites and pathogenic microorganisms such as bacteria and viruses (Krzystyniak et al., 1995; Ladics, 2007). The immune system also functions to efficiently eliminate any non-self components from within the body (Kidd, 2003). The immune system defends the host by initiating an innate immune response or acquired immune response (Delves and Roitt, 2000).

2.2.1 Cells, tissues and organs of the immune system

Various cell types are involved in the defense against invading pathogens. These cells originate and develop from pluripotent stem cells in the fetal liver and bone marrow (Delves and Roitt, 2000). Immune cells that are derived from a common myeloid, progenitor cell are termed polymorphonuclear leukocytes. Polymorphonuclear leukocytes include cell types such as neutrophils, eosinophils, mast cells and basophils. Mononuclear leukocytes include cell types such as monocytes, macrophages and lymphocytes. Immune cells that are derived from a common lymphoid progenitor cell are divided into either B or T lymphocytes (Eales, 1997).

The thymus and bone marrow are classified as primary lymphoid organs, where lymphocytes originate and develop. The spleen, lymph nodes, and mucosa associated lymphoid tissue (MALT) are referred to as secondary lymphoid organs and are lymphocyte-rich tissues in which B and T lymphocytes are activated (Pabst, 2007). Most secondary lymphoid organs have a specific structural design and microenvironment that facilitates increased communication between immune cells. This interaction between immune cells, initiates a specific and efficient immune response against invading pathogens (Randall et al., 2008).

B and T cells are both produced in the bone marrow and have to reach a stage of complete maturation before becoming part of the immune response. Maturation of B cells occurs in the bone marrow however, T cells have to migrate through the blood stream to the thymus where complete development occurs. Mature B and T cells first migrate to primary lymphoid organs and then progress to secondary lymphoid organs where they facilitate the elimination of an invading pathogen by an acquired immune response (Storni et al., 2005)

2.2.2 Mechanisms of the immune system

Innate system

The innate immune system operates as a primary line of defence against invading pathogens. This form of immunity has mechanisms in place that are always alert and equipped towards fighting against infectious agents and pathogens. The efficient mechanical and physical barriers offered by intact skin as well as the linings of the respiratory, gastro-intestinal and urogenital tract and chemical barriers such as antimicrobial proteins present a natural form of resistance to infective agents (Rollinghoff, 1997). The complement system also plays a very important part of the innate response by enhancing the process of phagocytosis (Mims et al., 2004). The innate immune system sets off protective reactions itself whereas the acquired immune response requires prior sensitization to an antigen (Ladics, 2007; Vos and Van Loveren, 1998).
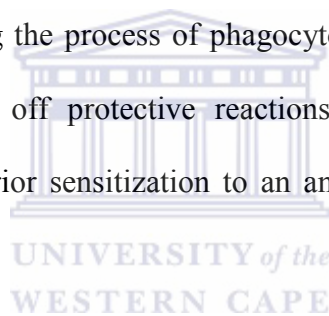
Acquired immune system

The acquired immune system is subdivided into two branches, namely the cell mediated and humoral immune responses. Both B and T lymphocytes function in recognizing and destroying invading pathogens and are the main cells involved in this form of immunity. An acquired immune response is reliant upon prior exposure to an antigen (Ladics, 2007; Nandi and Sarin, 2001).

Humoral immunity can be described in terms of the antibody producing B lymphocytes, which function in the regulation and manipulation of the humoral immune response (Storni et al., 2005). Humoral immunity functions in the synthesis of antibodies specific to antigens which eliminates pathogens via antibody-dependent

cellular cytotoxicity (ADCC) and initiates cell lysis mediated by the complement pathway or promotes phagocytosis that eradicates infectious agents (Ladics, 2007). Mature B cells possess certain surface molecules such as immunoglobulins (IgM and IgD) and accessory molecules (CD19, CD21, CD23 and CD37) that interact with viruses and bacteria. Antigen receptors, such as IgM and IgD have two functions in B cell stimulation. Firstly, when a cell attaches to an antigen, surface receptors transmit signals to the interior of the cell. This is followed by a second signal, which involves the transportation of the antigen to intracellular locations where it is processed and returned to the cell surface as peptides attached to Major Histocompatibility Complex class II molecules (MHC II). T helper cells identify and are stimulated by the MHC II-peptide complex to produce proteins (CD 40 ligand). These proteins in turn act on B cells stimulating them to produce antibodies (Storni et al., 2005).

Both cell mediated and humoral immunity are regulated by T lymphocytes (Kidd, 2003). Naïve CD8 T cells develop into cytotoxic T (Tc) cells that play a pivotal role in destroying virus-infected cells and are responsible for secreting cytokines that suppress the rate of viral replication (Storni et al., 2005). CD4+ T helper (Th) lymphocytes have a specific cytokine profile that enables them to be categorised into either Th1 or Th2 cells (Romagnani, 1996). Th1 and Th2 cells are derived from a non-committed group of precursor T cells. Direct communication of precursor T cells (naïve T cells), with antigen presenting cells (APCs) such as monocytes, macrophages, dendritic cells and others, initiates a differentiation process. This process of commitment is termed T cell polarization. The precursor T cells may enter a pre-activation state (T0) en route to differentiating into either Th1 or Th2 cells (Kidd, 2003). Stimulation of either a type 1 or 2 response is highly dependent on the type of cytokine occurring in the microenvironment of the T lymphocyte, especially

for the duration of antigen presentation and the commencement of the T cell response. Interleukin-12 (IL-12) and interferons (IFNs) are central in modulating the maturity of Th1 cells from naïve precursor T cells whereas IL-4 and IL-13 enhance the development of Th2 cells (Sinigalia and Ambrosio, 2000).

Th1 and Th2 cells are involved at a critical level of functioning in the immune system. Th1 cells initiate the cellular or type 1 pathway that functions in eliminating viruses and other intracellular pathogens, cancerous cells and promote delayed-type hypersensitivity skin responses. Th2 cells stimulate the humoral or type 2 pathway that increases antibody production used in the defence against extracellular pathogens. Over-stimulation of either the Th1 or Th2 pathway has been associated with the development of disease (Kidd, 2003).

2.2.3 Immunotoxicity

The unique nature or complex organization of the immune system has made it a target for chemicals or any foreign agents also referred to as xenobiotics (Krzystyniak et al., 1995). Therefore a potential disturbance by a xenobiotic at any level may lead to the immune system malfunctioning (Van Loveren and Vos, 1996). The harmful effects of a xenobiotic can result in different forms of toxicity (De Jong and Van Loveren, 2007). When the immune system becomes a target of chemical insults, the immunotoxicological result can be identified by a range of immune reactions such as immunosuppression or immunostimulation (Kunder, 2004).

Effects of immunotoxicity

Immunotoxicity can be induced either directly or indirectly. Effects of immunosuppression and immunostimulation are associated with directly induced

immunotoxicity. Indirectly induced immunotoxicity is associated with effects of immuno-hypersensitivity and autoimmunity (Van der Laan and Van Loveren, 2005). Some researchers view immunosuppression and immunostimulation as the two most important immune-mediated effects. Hypersensitivity reactions and autoimmunity are consequences of these immunotoxic effects (Anderson and Langone, 1999).

Immunosuppression

The maintenance of the functional integrity of the immune system is dependent on component cells and molecules, which are responsible for initiating an immune response that is effective in host resistance. Therefore, a disturbance in functionality of these component cells and molecules may lead to disease (International Programme of Chemical Safety (IPCS), 1996).

Immunosuppression may occur due to the impairment in antibody or T cell responses (Anderson and Langone, 1999) and as a consequence may lead to an increased susceptibility to infections as well as certain forms of neoplasia (Van Wijk and Nierkens, 2006). An apparent example of the effect of immuno-depression can be seen in the opportunistic infections that arise in HIV patients and cytomegalus infections in patients who have had organ transplants (De Jong and Van Loveren, 2007). Numerous cytostatic drugs such as, cyclophoshamide, cyclosporin A (CsA), prednisone and azathioprine are immunotoxic due to their immunosuppressive characteristics that unintentionally leads to numerous forms of cancers and an enhanced risk to either viral or a bacterial infections (Krzystyniak et al., 1995). CsA is a drug used extensively to diminish the rejection of an organ transplant and provokes a decline and deactivation of T cells (Carfi et al., 2007). It also causes impairment in the proliferative response of T cells and the decreased synthesis of

IFNγ, IL-1 and IL-2. Various *in vivo* studies with rats and mice show that the administration of CsA decreases the weight of the medulla of the thymus, alters splenic morphology and changes T cell production. These are manifestations that are all characteristic of immunotoxicity **(**Subcommittee on Immunotoxicology (Subcomm. on Immunotox), 1992). CsA and azathioprine are immunosuppressants that are used commonly in immunotoxicity studies specifically in the validation and assessment of functional immune assays (Snodin, 2004).

Exposure of animals and humans to environmental contaminants such as heavy metals can cause immunosuppression that result in reduced resistance to infections. In accordance with their immunosuppressive properties heavy metals are classified as follows: Mercury > Copper > Manganese > Cobalt > Cadmium > Chromium (Krzystyniak et al., 1995). Mercury is a highly, immunotoxic metal that has been given much attention due to its effect on the environment and on public health. Exposure of rodents to both organic and inorganic forms of mercury may result in immunotoxic effects. It has also been noted to functionally reduce the cell mediated response and is associated with the onset of autoimmune diseases (Silva et al., 2004). Other immunotoxic compounds that affect critical levels of functioning in the immune system are organotin compounds, polychlorinated biphenyls (PCBs), tetrachlorodibenzofuran (TCDF) and 2, 3, 7, 8-tetrachlor-dibenzodioxin (TCDD) which has reflected an immunosuppressive effect in experimental models (Krzystyniak et al., 1995).

The extent and effectiveness of invading pathogens as well as the degree of immunosuppression determines the type of infection that can occur in the body. The efficient immune system is normally responsible for defence against viruses; however,

immunosupppression facilitates viral-oncogene-dependent tumors. Tumors are likened to transplantation antigens that produce particular non-self markers on their exterior. The development of various tumors is common under immunosuppressive conditions **(**Subcomm. on Immunotox., 1992).

Immunostimulation

Directly induced stimulation of the immune system may lead to an exacerbated or lengthened immune response directed towards an invading pathogen. Chemically-induced immune dysfunction and drugs may also stimulate an enhanced immune response that enables hypersensitivity reactions and autoimmunity (Putman et al., 2003).

Hypersensitivity reactions

Most pharmaceuticals or metabolites need to covalently attach to carrier proteins in order to be converted into an efficient immunogen. This is followed by the formation of a Hapten-carrier complex, which is then detected by the adaptive immune system. The adaptive immune system functions in producing an immune response to any proteins that have changed due to the presence of a pharmaceutical or metabolite. These responses are termed hypersensitivity reactions (Van Wijk and Nierkens, 2006). In accordance with Gell and Coombs classification system, there are four classes of hypersensitivity reactions. Hypersensitivity reactions I, II and III are antibody mediated whereas T cells and macrophages mediate the hypersensitivity type IV response (Putman et al., 2003). A hypersensitivity reaction is a reaction to an antigen to which the host has been previously sensitized, having an adverse instead of protective effect. The most frequent types of immunotoxic reactions are

hypersensitivity type I and type IV. Hypersensitivity type I or anaphylactic reaction is IgE mediated and initiates the release of mediators such as histamine from basophils and mast cells. Type IV (delayed hypersensitivity reaction) responses affect stimulated T cells which generate cytokines as well as other mediators. As a consequence of this reaction, cellular and tissue damage may occur (Anderson and Langone, 1999). Hypersensitivity type IV reactions appear as skin eruptions on the body (Van Wijk and Nierkens, 2006). This type of reaction also manifests as allergic contact dermatitis due to drug and environmental exposure (Putman et al., 2003).

Persistent chronic inflammation

Enhancement of an immune response may lead to prolonged inflammatory reactions (Putman et al., 2003). Persistent chronic inflammation is considered an immunotoxic effect. Inflammation is a normal response to injury or damage at a site in the host. Acute inflammation attracts several neutrophils as the main group of cells at the site of injury and this is a short term response. Healing of a lesion normally involves acute inflammation followed by chronic inflammation. However, when there is an immunotoxic effect of a xenobiotic on the immune system, then chronic inflammation may become persistent. This is a major problem since the consequences are granuloma formation, various detrimental immune reactions and autoimmunity can occur. Therefore, it is vital to be able to distinguish between short term chronic inflammation, which is the body's normal response to injury and prolonged chronic inflammation, which has a negative effect on the immune response (Anderson and Langone, 1999).

Autoimmunity

Diseases caused by an immune response directed towards self antigens are termed autoimmune diseases (Van Loveren et al., 1995). Autoimmunity may be the consequence of changes in T cells, which are involved in the modulation of cell mediated and humoral responses (Gutcher and Burkhard, 2007; Kidd, 2003). Numerous environmental chemicals or pollutants are most likely to be causal factors that contribute to the development of autoimmune diseases (Inadera, 2006). In the case of drug induced autoimmune effects, the immune system is exposed to unfamiliar self epitopes and may be transformed due to the effect of the drug. Autoimmune reactions that are drug induced are extremely idiosyncratic and therefore the availability of specific drugs for consumer purchase has been stopped (Van Wijk and Nierkens, 2006).

Autoimmunity includes a wide range of manifestations such as Hashimoto's thyroiditis, systemic lupus erythematosus (SLE), Guillain-Barré syndrome, Sjögren syndrome, rheumatoid arthritis and autoimmune haemolytic anemia (Bussone and Mouthon, 2009). Multiple sclerosis and rheumatoid arthritis are autoimmune diseases mediated by T lymphocytes and are stimulated by auto-aggressive T helper cells. It has been commonly believed that T helper cells have a direct role in these diseases but it should also be taken into consideration that antigen presenting cells are important participants in the onset and development of an autoimmune response (Gutcher and Burkhard, 2007).

2.2.4 Evaluation of immunotoxity

In determining the effect of xenobiotics on the immune system it has been established that the most suitable approach to test for immunotoxicity is a 'tier' approach (De Jong and Van Loveren, 2007). Due to the complexity and varying nature of the immune system, the tier testing procedure has been recommended and this approach has been authenticated in inter-laboratory studies with rats and mice. The tier testing model for rats and mice has proven to be an effective indicator of immune impairment due to xenobiotic exposure. This procedure has also been effectively applied in other animal species such as non-human primates (Vos and Van Loveren, 1998). The determination of potential immunotoxicity of a chemical compound on the immune system for a species is conducted according to the Organisation of Economic Cooperation for Development (OECD) guideline, which consists of bioassays after acute, short-term and long-term exposure to chemicals. This testing procedure consists of tier 1, which is a general toxicity study that focuses predominantly on the evaluation of organs of the immune system and tier 2 which consists of more specific tests that includes host resistance or in depth mechanistic studies (Krzystyniak et al., 1995).

Tier 1 (General toxicity study)

Tier 1 assesses alterations in lymphoid tissue and immune cell populations. This is done in accordance with the toxicity endpoints and health condition of the test animal (Van der Laan and Van Loveren, 2005).

Some indicators or common endpoints of immune toxicity for tier 1 include hematology, organ and body weight indicators as well as the histological evaluation of

lymphoid organs such as the spleen, thymus, lymph nodes, Peyer's patches and bone marrow (Van Wijk and Nierkens, 2006). The histopathological evaluation of lymphoid organs has been shown to be a reliable indicator for determining immunosuppression while changes in thymus and spleen weights are very dependable indicators of systemic imunotoxicity (De Jong and Van Loveren, 2007). The histopathogical examination of lymphoid tissue enables the recognition of alterations seen in tissue architecture due to xenobiotic exposure, however it is inadequate in determining alterations in cell numbers. It is therefore, recommended that hematology is taken into consideration when assessing the histopathology of lymphoid tissue (Basketter et al., 1995).

Tier 2 (Specific testing)

Assays performed in the second tier, evaluate effects of immunotoxicity on the immune system more comprehensively (De Jong and Van Loveren, 2007). The second tier studies are required when there are indications in the first tier of adverse effects (Van der Laan and Van Loveren, 2005).

In tier 2 functional assays such as such as macrophage activity, natural killer cell activity, mitogen induced B and T cell proliferation and delayed hypersensitivity (DTH) responses are usually performed (De Jong and Van Loveren, 2007). Functional assays together with other quantitative techniques may prove to be very useful tools in determining the type of cell function that has become the focus of immunotoxic chemicals (Krzystniak et al., 1995). The Colony Forming Unit-Granulocyte /Macrophage (CFU-GM) assay is used as a screening test to determine chemically stimulated myleosuppression and is considered to be a validated *in vitro* approach (Carfi et al., 2007). This colony forming or methylcellulose assay is the

most employed *in vitro* assay for the quantification of lineage committed haematopoietic progenitors (Colony Forming Unit-Eosinophil, CFU-Granulocyte, CFU- megakaryocyte etc). The end point of the CFU-GM assay is inhibition of cell proliferation by colony scoring. If the cells are viable then the functionality of these cells can also be determined. Cell proliferation can be investigated with the use of mitogens such as phytoheamaggluttinin (PHA), concavalin A (Con A) and, anti-CD3 or anti-CD28 antibodies (especially for T cells). Similarly, cytokine synthesis and its quantitative variations can also be used as an indicator of immunotoxicity (Carfi et al., 2007).

In tier 2 the immune responses to various types of antigens like the tetanus toxoid and ovalbumin, sheep red blood cells (secondary immune response to SRBCs) and T cell independent antigens such as lipopolysaccharide (LPS) are also investigated (De Jong and Van Loveren, 2007). Host resistance models as tier 2 assays allow the measurement of end points that are clinically significant and relevant. These models are thus important and provide information that enables scientists to predict health risks associated with xenobiotics (Van Loveren et al., 1995).

Dose levels and toxicity

The choice of a dose concentration when conducting an immunotoxic study is crucial as it affects the end result. Non-specific stress responses may cause a test compound to be incorrectly identified as immunotoxic. Dose concentrations that allow for clear dose-response curves and no-observable-effect level (NOEL) should be chosen. In certain cases, selecting dose concentrations that stimulates overt toxicity may be helpful; however, any immune alteration noticed at that particular dose level should be carefully interpreted. This may be due to malnutrition or serious stresses that may

cause inefficient immune responses (Snodin, 2004). The current study uses a combination of tier 1 and tier 2 approaches to effectively evaluate the effects of both natural and artificial sweeteners on specific immune pathways.

Research determining the effect of artificial (aspartame, saccharin and sucralose) and natural sweeteners (brown sugar, white sugar and sugar cane molasses) on the immune system is limited. However, anecdotal evidence does suggest that these sweeteners may have the potential to induce adverse effects on human health (Kovacs, 1996-2011). The immune system is central in ensuring homeostasis within the body and therefore it is necessary to determine the potential impact of such sweeteners on this system.

2.3 The reproductive system

The endocrine system is an important component of the body that functions in the growth, development, reproductive activities and behaviour of both humans and wildlife (Caserta et al., 2008). Since World War II, large amounts of chemicals synthesised by industries as well as other endocrine-active compounds have been released into the environment causing adverse effects on animals and humans. Such adverse effects include reduction in sperm counts, reduced ability of male reproduction and various morphological and hormonally associated deformities (Safe, 2000). The risks associated with exposure to endocrine disruptors have become both the focus of scientific debate and of interest to the general public over the past decade. This topic continues to remain as a major issue today (Solomn and Schettler, 2000).

2.3.1 Endocrine disruptors and the endocrine system

An endocrine disruptor may be defined as "an exogenous substance or mixture that alters the functions of the endocrine system and as a result induces adverse health effects in an intact organism, or its progeny, or (sub) populations" (International Programme on Chemical Safety IPCS, 2002). Endocrine disrupting compounds (EDCs) are widespread in the environment (air, land and water) and in food products of animal or plant sources. Combustion products, food packaging, detergents and health remedies using plants are sources from which EDCs may originate (Mostafa et al., 2007). A wide array of other substances such as synthetic chemicals (Dichlorodiphenyl-trichloroethane (DDT) and associated compounds, polychlorinated biphenyls (PCBs) and dioxins, industrial chemicals (alkyphenols and phthlalates) and naturally occurring phytoestrogens (soybeans, grains and carrots) are examples of

potential endocrine disruptors (Society of Environmental Toxicology and Chemistry (SETAC), 2000).

The endocrine system is made up of various organs such as the thyroid, pancreas, pituitary and reproductive organs that function in manufacturing hormones (Hollander et al., 1997). Hormones communicate with receptors at both a cellular and nuclear level as well as in tissues and organs. These interactions initiate specific biochemical responses that are essential in the maintenance of homeostasis within the body. Therefore, any interference caused by EDCs may induce injury to the physiological status of the entire organism (Caserta et al., 2008). Various EDCs have mechanisms by which they disrupt normal hormonal action. This includes imitating natural hormones, blocking hormone receptors as well as initiating change in the metabolism of endogenous hormones (DiDiego et al., 2005). Other EDCs act by attaching to carrier proteins, which hinders the accessibility of carrier proteins to convey hormones via the bloodstream.

2.3.2 Reproductive toxicity

Reproductive toxicity may be defined as the adverse impact of a compound on the reproductive cycle. Such adverse effects include the ineffective function of the reproductive system, growth retardation, abnormalities or even death (Mantovani and Maranghi, 2005). Various chemicals, commercial products as well as environmental contaminants have been associated with an increased susceptibility to hormone-dependent cancers as well as unfavourable effects on sexual differentiation (Sanderson, 2006). The endocrine system is largely responsible for the maintenance of reproductive fitness and therefore a disturbance induced by an exogenous agent may affect reproductive development (Mantovani and Maranghi, 2005).

2.3.3 The male reproductive system

There are various sites of the male reproductive system that endocrine disruptors target. These areas mainly include the testes and male gonads, which are locations of androgen synthesis and spermatogenesis (Sikka and Wang, 2008). The testis functions in synthesising fertile sperm and steroid hormones that are necessary for reproducibility and sexual function (Sanderson, 2006). Spermatogenesis occurs in the seminiferous tubules, which constitutes approximately 80 % of the testicular mass. Leydig cells and Sertoli cells make up the other 20 % and these cells function in maintaining normal spermatogenesis (Sikka and Wang, 2008). Maturation of the male reproductive system is a complex process that is dependent upon the communication of various hormones and factors. The androgens, testosterone and dihytestosterone are vital factors necessary for the growth of both the internal and external male reproductive system. During the neonatal and fetal stages androgens that are synthesised, aid in maintaining the wolffian duct that differentiates into the epididymis, vas deferens and seminal vesicles. Testosterone is responsible for the masculinization of the above-mentioned reproductive structures, while dihytestosterone is responsible for the masculinization of the external genitals and prostate. The pivotal role played by androgens in the development of the male reproductive tract is the target of many chemicals, which have the ability to disrupt the activity or production of androgens. This may lead to devastating effects on the developing male reproductive system (Fisher et al., 2004).

The effect of endocrine disruptors on the male reproductive system

The adverse effect of pesticides on the human health was initially recognised in 1960; however, it is only lately that prolonged exposure in low doses to pesticides has been

associated to effects such as reproductive malformations, cancer, immunosuppression and endocrine disruption (Srinivasa et al., 2005). Pesticides are a wide range of chemicals that function to eliminate insects, weeds, rodents and fungi. These chemicals have proven to be very beneficial in the agricultural industry; however, it has also been shown that pesticides pose a major risk to human health. This is evident from the statistics that reveal approximately 5 million people are poisoned globally by pesticides (Jurewicz et al., 2006).

Dichlorodiphenyl-trichloroethane (DDT), kepone, epichlorhydrin, dioxin and ethylene dibromide are examples of agricultural pesticides that induce reproductive toxicity in males (Sikka and Wang, 2008). Research has shown that DDT exposure in males have been associated with decreased sperm counts, low semen volume following ejaculation and decreased serum testosterone levels. Due to both the estrogenic and anti-androgenic potential of DDT, this pesticide has been removed from the market in many countries. However, a few developing countries continue to use DDT to eradicate insects causing diseases such as malaria (Gunnarsson, 2008).

Compounds such as glycol ethers, 2, 5 hexanedione and benzimidazole fungicides have a direct impact on the proliferation and differentiation of seminiferous epithelium and may therefore induce male reproductive toxicity. There is increasingly more focus on toxicity mechanisms that affect the regulation of spermatogenesis. This is a result of several compounds proving to be toxic to main cell types involved in spermatogenesis. Ethane dimethane sulfonate is an example of such a chemical that has a direct effect on Leydig cells in adult rodents, which acts in the regulation of testosterone production (Mantovani and Maranghi, 2005). Sertoli cells form tight junctions that make up a blood testis barrier, eliminate defective

sperm and produce the hormone inhibin that modulates the production of sperm. Therefore, these cells prove to be crucial participants in the process of spermatogenesis (Sikka and Wang, 2008). *In vitro* studies have shown that chlorinated insecticides, cadmium chloride, bisphenol A and dinitrobenzene have a direct impact on Sertoli cells by targeting Sertoli tight junctions. These chemicals act by decreasing the quantity or inducing the intracellular localization of junctional proteins, thus affecting the process of spermatogenesis (Mantovani and Maranghi, 2005).

The process of testicular steroidogenesis consists of essential enzymes that act in the biosynthesis of numerous steroid and sex hormones. Endocrine disrupting chemicals have made these key enzymes targets of their action (Sanderson, 2006). Increasingly, research has been focusing on the enzyme aromatase (CYP19) which functions in the conversion of androgens to estrogens (Whitehead and Rice, 2006). Azole fungicides such as prochloraz and imazalil are examples of compounds that inhibit aromatase activity *in vitro*. *In vivo* effects were demonstrated in a study that investigated aromatase inhibition by the fungicide, fadrozole on embryonic growth in chicken eggs. Results showed that suppression of aromatase activity lead to masculinization of female embryos. A follow-up study revealed female hatchlings that were exposed to fadrozole, developed gonads similar to testes with the occurrence of atypical seminiferous tubules (Sanderson, 2006).

Bisphenols, phthlalates and alkylphenols (AP) are the three main industrial endocrine disruptors that have recently become the focus of interest to the general public (Gultekin and Ince, 2007). Bisphenol A (BPA) is used extensively in the manufacture of polycarbonate plastics, resins, dental sealants as well as in food and beverage

containers (Maffini et al., 2006; Mostafa et al., 2007). Research investigating the extent of BPA exposure among the human population show that levels of BPA can be detected in serum of both adult males and females, in maternal and fetal plasma, in the milk of nursing mothers as well as in amniotic fluid of the fetus. BPA induces low sperm counts and a high incidence of hypospadias, cryptorchidism, testicular cancer, breast cancer and brain tumors (Maffini et al., 2006). On the other hand, AP is used in the manufacture of alkylphenol polyethoxylates (APEOs), which enters the market as nonylphenol polyethoxylates (NPEOs). NPEOs are used largely in the manufacture of plastics, paints, pesticides, agricultural chemicals, cosmetics and household detergents. It is therefore evident that a large portion of the human population is exposed to this chemical on a regular basis. NPEOs gain access into the environment via waste water streams and under anaerobic surroundings (sewers, sediments and biotreatment operations) they are oxidised to nonylphenols (NPs) (Gultekin and Ince, 2007). NP is well known for its toxic effect on acquatic life and for its estrogenic potential. It has been hypothesised that pesticide spraying containing NPs is responsible for the reduction seen in salmon numbers in Atlantic Canada as well as for the estrogenised effects of fish in British streams (Safe, 2000). Dermal exposure and inhalation appear as the most important routes of phthalate exposure as a result of their broad use in the manufacture of plasticizers as well as in cosmetics and insect repellents. Phthalate toxicity is linked to adverse health effects such as malformations in the male reproductive system, testicular and breast cancer as well as neuro-endocrine disruption (Gultekin and Ince, 2007).

Endocrine disruptors that appear as estrogen like or anti-androgenic in the environment may also have severe effects such as infertility and erectile dysfunction on the male reproductive system. Other sources of exposure besides xenoestrogens

(man made chemicals), include phytoestrogens which are non steroidal compounds manufactured by plants and mycoestrogens such as zearalenone, which are metabolites from fungi species. Mycoestrogens present in grain have shown the ability to induce estrogenizing syndromes in both cattle and poultry, while the estrogenising effects of phytoestrogens have shown to cause infertlility in Australian ewes (Vidaeff and Sever, 2005). Normal endocrine functions can be affected due to numerous synthetic pharmacological products and phytoestrogens, which have lead to symptoms of oligozoospermia and a reduction in libido in human males (Sikka and Wang, 2008).

2.3.4 The female reproductive system

The female reproductive tract is a complex system that is dependent upon homeostasis and normal hormonal levels that are necessary to maintain growth and development (Stamati and Pitsos, 2001). Ovarian steroidogenesis is a process that facilitates folliculogenesis, ovulation, and pregnancy. Therefore, female reproductive health is highly dependent on the management of this process. Endogenous growth factors, steroid hormones, cytokines as well as exogenous agents function in the control of both ovarian maturation and function (Uzumcu and Zachow, 2007). It has been hypothesised that exposure to endogenous factors during early development stages causes an individual to be susceptible to the progression of disease in later life (Gore et al., 2006). Pre-natal and early post-natal stages are most at risk to endocrine disruption due to the rapid change occurring in organ and neural systems. Individuals are also more susceptible to endocrine disruption during puberty and peri-menopausal phases when variations of hormonal effects occur (Mostafa et al., 2007). Exogenous factors such as, environmental agents possess estrogenic or anti-estrogenic,

androgenic or anti-androgenic effects that may act by interfering with the ovarian cycle. This has a devastating consequence by adversely affecting female fertility (Caserta et al., 2008). Other adverse health effects include abnormalities of the menstrual cycle, endometriosis and polycystic ovarian syndrome of the female reproductive tract (Stamati and Pitsos, 2001).

The effect of endocrine disruptors on the female reproductive system

Most endocrine disrupting chemicals that occur in the environment are comprised chemically of carbon and chlorine. These chemicals are termed organochlorines. Organochlorines possess a lengthy half life due to the resistance against degradation by biochemical and physical processes. Both wildlife and humans are unable to detoxify or eliminate these substances and as a result organochlorines accumulate in fatty tissue and lipids. These substances act by interfering with hormonal homeostasis within the body or estrogen activity (Stamati and Pitsos, 2001). Therefore, these chemicals may participate in advancing puberty or breast development or postponing menopause in females (McLachlan et al., 2006). Reproductive toxicities have been associated with organochlorine compounds such as PCBs, polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (Safe et al., 2000).

Pesticides such as dichlorodiphenyltrichloroethane (DDT) or 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) and PCBs are persistent organochlorine contaminants that occur in the environment at low concentrations, but still remain the largest percentage of compounds that have been detected in human and wildlife samples (Safe et al., 2000). There is considerable evidence relating occupational exposure to pesticides with infertility in males. However, amongst women such research studies are limited. This is probably due to the higher pesticide exposure rate of men to

women or because fertility in females is a more complex process to evaluate than in males (Bretveld et al., 2006). Farr et al. (2004) reports that approximately 60-100 % of females exposed to pesticides suffer from a lack or increase in the duration of the menstrual cycle, or intermenstrual bleeding when compared to women that were not subjected to exposure (Farr et al., 2004).

Endometriosis is a common gynaecological disorder characterised by the occurrence of endometrial glands and stroma on the exterior of the uterine cavity. This is an estrogen dependent disorder and a major cause factor of female infertility (Caserta et al., 2008; Chedid et al., 1995).   Studies investigating the effect of 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) and PCBs on the female reproductive tract of monkeys found that these compounds strongly induce endometriosis. High levels of PCBs were detected in serum levels of monkeys and the concentrations of TCDD detected in exposed animals were similar to those found in the serum, milk, and tissues of humans. Therefore, such findings may be applicable to that of the human population (Caserta et al., 2008). It has been suggested that TCDD may have an impact on granulosa cells that function in the synthesis of steroid hormones, which play a pivotal role in the ovarian cycle. TCDD may act on human luteinized granulosa cells by inhibiting the mitotic signal either directly or indirectly.

Phthalates are synthetic chemicals used primarily in the manufacture of plastics and various other consumer products. As a result, the extent of their exposure to humans is vast. Di-(2-ethylhexyl) phthalate (DEHP) has been reported as one of the most toxic phthalates to act on the reproductive system.   *In vivo* studies with female rodents revealed that DEHP targets the ovary and decreases the synthesis of estradiol. It has been shown that rats exposed to DEHP suffer from low serum estradiol levels,

delayed estrous cycles, and the lack of ovulations. In addition, the lack of ovulations results in the absence of the corpora lutea, which contributes to follicles becoming cystic. The morphometric examination of the pre-ovulatory follicles showed that granulosa cells of DEHP-exposed rats were considerably reduced compared to control cells. Thus, from the above mentioned it can be determined that granulosa cells are the target cells of DEHP in the ovary (Lovekamp-Swan and Davis, 2003).

Numerous, synthetic pharmacological agents as well phytoestrogens adversely affect endocrine functions in a healthy individual (Sikka and Wang, 2008). Diethylstilbestrol (DES) is one the most recognised synthetic drugs produced. During the period of 1940-1950's, DES was prescribed to pregnant women. The outcome of this prescription was detrimental leading to the development of breast cancer, endometriosis, menstrual irregularities, premature delivery and miscarriage in these individuals. Girls born to these pregnant women were at risk of developing vaginal clear-cell adenocarcinoma, a type of cancer that occurs in 50 year old women but may be accelerated in 30 year old females who were exposed to DES in utero (Hollander, 1997, Stamati and Pitsos, 2001).

Breast and endometrial cancer has gradually increased since the 1940's in various industralised countries. Dietary factors have been identified as one of the contributing factors of breast cancer. Phytoestrogens is a specific group of dietary compounds that has received a great deal of attention (Caserta et al., 2008). Phytoestrogens consist of isoflavones, lignins, coumestan or resorcylic acid lactones which appear to be structurally alike to the mammalian estrogen. Human consumption of phytoestrogens is sourced mainly from soybeans (Greim et al., 2004). Although phytoestrogens affect endogenous hormone concentrations, their participation in breast cancer

initiation and progression remains unclear. Dietary consumption of phytoestrogens may be beneficial against breast cancer, cardiovascular disease and post-menopausal disorders; however, a high intake of soy products during pregnancy or post-natal stages is of major concern. Miodini et al. (1999) demonstrated using human mammary epithelial cells (MCF-7) that genistein, a soy isoflavone, at low concentrations can induce cell proliferation while at high concentrations can function as an inhibitor (Miodini et al. 1999). Factors such as tumour cell type, concentrations, type of phytoestrogen or time of exposure may contribute to both the proliferative or anti-proliferative effects of genistein. Other phytoestrogens besides geinstein may act by inhibiting the action of the aromatase enzymes that function in converting androgens to estrogens. Research has also shown that phytoestrogens at higher concentrations have the potential to initiate apoptosis in breast cancer cells as well as possess antioxidant properties that activate the immune system and inhibit angiogenesis (Caserta et al., 2008).
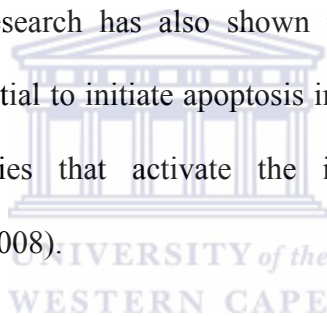
This study aims to evaluate the effects of certain nutritive and non-nutritive sweeteners on the male and female reproductive systems. Artificial and natural sweeteners have had a long history of use and have become common dietary products. However, anecdotal reports suggest that these sweeteners may have the potential to affect different organ systems (Kovacs, 1996-2011; VEM, 2000). However, there is a lack of data that directly determines the effects of artificial and natural sweeteners on the reproductive system. The reproductive system is a common target of potential endocrine disruption and therefore it is necessary to determine the impact of such sweeteners on this organ system.

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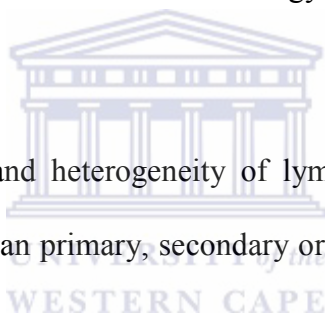
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Chapter 3

The *in vitro* effects of artificial and natural sweeteners on the immune system using whole blood culture assays

Sections of this chapter have been published or submitted for publication:

1. Rahiman F, Pool EJ (2010). The effects of *Saccharum officinarium* (sugar cane) molasses on cytokine secretion by human blood cultures. J. Immunoassay Immunochem. 31(2): 148-159.

2. Rahiman F, Pool EJ (2011). The *in vitro* effects of artificial and natural sweeteners on the immune system using whole blood culture assays. J. Food Sci. (Submitted online on 28th April 2011).

Chapter 3

3.1 Abstract

This study investigated the effects of commercially available artificial (aspartame, saccharin, sucralose) and natural sweeteners (brown sugar, white sugar, molasses) on the immune system. Whole blood cultures were used as a model system and harvested supernatants were assayed for cytotoxicity and cytokine release. Results show that no artificial or natural sweeteners induced cell death when incubated with whole blood cultures. The natural sweetener, sugar cane molasses (12.5–800 μg/ml) increased the synthesis of the inflammatory biomarker, Interleukin-6 as well as the biomarker of humoral immunity, Interleukin-10 at concentrations ranging from 200 μg/ml to 800 μg/ml. This suggests that molasses elevates inflammatory activity and increases humoral immunity. Humoral immunity is used in defences against extracellular pathogens. In a comparative experiment, molasses (10 μg/ml) demonstrated its ability to enhance the process of inflammation, while artificial sweeteners revealed a suppressive effect on the inflammatory response (10 μg/ml). Exposure of sucralose-containing sweeteners to blood cultures reduced levels of Interleukin-10 under stimulatory conditions. The suppression of Interleukin-6 and Interleukin-10 levels induced by sucralose, indicate a potential suppression of humoral reponses.

3.2 Introduction

The desire for sweet tasting food is inherent and characteristic of the human population. Prehistoric man satisfied his need for sweet taste through the intake of certain fruits and vegetables. Years later, through the advancement in food technology, refined sugar was made accessible to people at low cost. This sparked an increase in the use of sugar worldwide (Sardesai and Waldshan, 1991).

Today, there is an extensive choice of sweeteners available to the consumer. These sweeteners can be categorised into either nutritive (natural sweeteners) or non-nutritive sweeteners (artificially manufactured sweeteners). Sugar cane molasses is an example of a popular, natural sweetener rich in sucrose and is the by-product of the sugar refinement process (Greenly, 2003). Dating back to the nineteenth century, molasses has been used widely in livestock and poultry feeds (Curtin, 1983). Today, molasses is increasingly being used as a flavour enhancer, has been substituted as a sweetener and used as a preservative in jams and jellies (Reyed and El-Diwany, 2008). Anecdotal reports also suggest that molasses may be used as a supplement in the human diet to improve conditions such as anaemia, colds, coughs, earaches, arthritis, ulcers, hair damage, eczema, high blood pressure, dermatitis, constipation, varicose veins, nerve damage, and bladder problems (Kirschmann, 2007; Reyed and El-Diwany, 2008; Crellin et al., 1990).

Although molasses has been associated with various health benefits, there are also reports that suggest the inclusion of molasses in the diet of livestock may induce certain metabolic diseases. Such diseases include molasses toxicity, urea toxicity and bloat which may occur as a result of molasses being used as a supplement (vehicle for urea) or as the basis of livestock feed (Preston et al., 1986). Molasses toxicity is

defined as a condition affecting cattle or sheep fed high molasses diets with limited forage (Lora et al., 1977). Affected animals suffer from symptoms similar to that of cerebro-cortical necrosis or polioencephalomalacia. Bloat is a condition characterised by the retention of gas in the rumen and occurs in most animal feeding systems. However, this disease appears to be recurrent in diets consisting of carbohydrates supplied by unrefined sugar or maize grain that has little or no fibre, yet is easily digestible (Preston et al., 1986).

Sugar or sucrose consumption has been associated with dental caries, obesity and chronic diseases linked to obesity such as cancer, diabetes and heart disease. As a result, this created a demand for the development of alternate sweeteners, which offer a low calorie intake (Sardesai and Waldshan, 1991). The manufacture of artificial sweeteners by the food industry, promised consumers the sweetness of sugar without the calories (Whitehouse et al., 2008). Diabetic patients and individuals wanting to control their weight have access to these products that allow them the pleasure of a sweet-tasting treat without adverse health effects (Sardesai and Waldshan, 1991). These alternate sweeteners are used extensively as additives in food, beverages, confectionary, and also in the pharmaceutical sector. Common types of artificial sweeteners include aspartame, acesulfame-K, cyclamate, neotame, sucralose and saccharin (Zygler et al., 2009). Although artificial sweeteners have been welcomed into the food industry for its beneficial uses, some studies have found correlations between their use and illnesses such as cancers, hepatotoxicity, headaches, allergies, seizures, diarrhoea and low birth weight (Whitehouse et al., 2008).

Commercially available saccharin is almost 300-500 times sweeter than sucrose (Arnold et al., 1983). This artificial sweetener is probably one of the most

scrupulously researched sweeteners since its discovery, with much of the focus pertaining to its potential role in bladder cancer (Greenly, 2003). There have been approximately 20 experimental groups that have independently investigated the effect of elevated doses of saccharin in one-generation rats. Results from these studies show that the majority of these groups could not positively associate saccharin-fed rats with the development of neoplasias. Although the positive control group did reflect an increased incidence of bladder cancer, this result was questionable since rats that were used in the studies were regularly infected with the bladder parasite *Trichosomoides crassicanda*. In two-generation rat studies, results show almost consistently that rats fed on saccharin diets and born of saccharin-fed parents had an increased risk of developing bladder cancer (Weihrauch and Diehl, 2004).

Aspartame first came into existence in 1965 and has been packaged under labels such as 'Nutra sweet', 'Canderel' and 'Equal' (Szucs et al., 1986; Grenby, 1991). In the body, aspartame is hydrolysed into its components namely, phenylalanine, aspartic acid and methanol. Research has focused on the plasma levels of these products within the human body. These studies have revealed both potentially normal conditions of use and adverse effects arising from the consumption of aspartame (Renwick, 1985). In 1996, Olney and colleagues published a contentious paper suggesting that a link between the surge in brain tumors since 1980 and the introduction of aspartame into the food industry existed. They further supported their hypothesis by referring to an FDA experiment in which 12 out of 320 Sprague-Dawley rats developed malignant brain tumors following long-term, aspartame ingestion. This hypothesis raised much concern amongst the general public but was also profoundly criticised by many scientists (Weihrauch and Diehl, 2004; Olney et al., 1996).

Sucralose has been branded under the name 'Splenda' and has a wide variety of uses in the food industry (Whitehouse et al., 2008). The production of sucralose involves the replacement of three chlorine atoms for three hydroxyl groups in sucrose. The safety concern of this compound arises as a result of the existence of three chlorine atoms, which make it an organochloride. Organochlorides such as pesticides and dioxins have been largely reported as being carcinogenic. Hence, sucralose has also been subjected to toxicity screening (Zygler et al., 2009).

Both natural and artificial sweeteners have become well established in the food industry and therefore we all have a vested stake concerning its safe use. The movement from the use of sucrose to artificial sweeteners still remains a controversial topic. The demands for both natural and synthetic sweeteners have increased enormously in this decade. Therefore, it is necessary that these sweeteners are subjected to regular toxicity testing and this should remain under constant evaluation since new products are continually being developed.

This study aimed to determine the potential adverse effects of natural and artificial sweeteners on the immune system. In order to achieve this aim, we had two main objectives. Firstly, we investigated if commercially produced sugar cane molasses affects specific cytokines regulating the immune pathway. Based on the outcome of this objective, we aimed to further investigate the comparative effects of both natural (sugar cane molasses, brown sugar and white sugar) and artificial sweeteners (Canderel™, Equal™, Natreen™, Sweetex™, Splenda™ and Swheet™) on the immune system.

The immune system is an intricate and interactive network that comprises of various components. This system primarily functions in defense against foreign or antigenic

particles entering the body as well as protecting the integrity of the host against attack and disease triggered by pathogenic organisms (Pillai and Watson, 1990). The human immune response consists of two branches which includes innate and acquired immunity. Phagocytic cells and cellular components such as interferons make up the innate immune response, which acts as the first line of defense against pathogens (Ladics, 2007; Nandi and Sarin, 2001). The acquired immune response consists of two branches, namely cell mediated and humoral immunity. Both B and T lymphocytes function in recognizing and destroying invading pathogens and are the main cells involved in this form of immunity. The acquired immune response requires prior sensitization to an antigen whereas the innate immune response is not dependent on exposure to an antigen (Ladics, 2007).

Research in toxicology has shown that the immune system is a target for various chemicals and that a toxicant has the ability to affect a constituent of the immune system at any level (Burrell, 1993; Vos et al., 1989). Immunotoxicology can be defined as 'the study of adverse effects of foreign substances also referred to as xenobiotics on the immune system, which results in harmful alterations in the host responses and ultimately leads to the increased risk to infectious disease' (Burrell, 1993). A chemical insult to the immune system encompasses a variety of effects that include enhancement or suppression of the immune response (Colosio et al., 2005). A decreased immune response may be associated with an increase in the incidence, time-span, severity or result of an attack by an invading pathogen. An enhanced immune response may be either beneficial or detrimental to the host since it may further increase the immune response to a disease or either neutralise the effects of autoimmunity or hypersensitivity reactions (Pillai and Watson, 1990). Industralised countries are experiencing a significant increase in diseases that can be associated

with a malfunction in the immune system (Colosio et al., 2005). Thus, the immunotoxicity of drugs and various compounds, particularly those that we are exposed to regularly, are progressively being identified as potential hazards (Langezaal et al., 2001).

Since their existence, the uses of natural and artificial sweeteners have been quite controversial. Their implication in various diseases has raised much questionability with respect to their safe use (Whitehouse et al., 2008). Hence, this study aims to determine the potential adverse effects of both natural and artificial sweeteners on the immune system. Whole blood cultures (WBCs) were used as a model system to determine the impact of sweeteners on immune function. Lactate dehydrogenase release was used as a biomarker for cytotoxicity. Interleukin-6 was used as a biomarker of the inflammatory response, Interleukin-10 as a biomarker of humoral immunity and Interferon-gamma as a biomarker of cell mediated immunity.
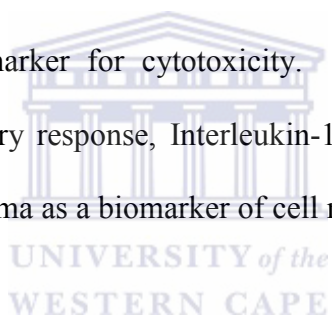
3.3 Materials and methods

3.3.1 Principle of the procedure

In this study, human blood was treated with the B-cell mitogen, lipopolysaccharide (LPS) and the T cell mitogen, phytohemagglutinin (PHA). LPS stimulates an inflammatory response and initiates the release of interleukin-6 (IL-6) from monocytes and B lymphocytes. PHA was used to stimulate the cell mediated and humoral immune pathways, initiating the release of interferon-gamma (IFN-γ) and Interleukin-10 (IL-10) from T lymphocytes (Hussain et al., 2002). Cytokine production was measured in the supernatants, following incubation with or without the test compound. Cytokines were quantified using enzyme linked immunosorbent assays (ELISAs).
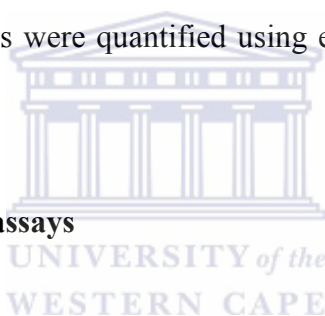
3.3.2 Whole blood cytokine assays

Blood was obtained from healthy, male volunteers and stored at ambient room temperature. Consent was obtained from all participants. Blood samples were collected in 10 ml citrate-containing vacuum tubes by venipuncture and used within 8 hours of collection. All procedures were performed under sterile conditions. Whole blood was diluted 1:10 with Roswell Park Memorial Institute (RPMI)-1640 (Sigma-Aldrich, Germany) before being stimulated or not stimulated with a mitogen.

3.3.3 The effect of molasses samples on endotoxin stimulated and unstimulated whole blood cultures (WBCs)

Stimulated whole blood cultures contained 1 volume of 10 ng/ml endotoxin in DMSO, 10 volumes of blood and 89 volumes of RPMI-1640 medium (Sigma-

Aldrich, USA). Unstimulated blood contained 1 volume DMSO, 10 volumes of blood and 89 volumes of RPMI-1640. Dilution ranges of molasses (Health Connections Wholefoods, SA) in distilled water was dispensed at 3 µl/well in wells of 96-well plates (Nunc-Immuno plate, Serving Life Science, Denmark). Endotoxin stimulated or unstimulated diluted blood (300 µl/well) was added to molasses samples and thereafter incubated at 37 °C for 18 hours. At the end of the incubation period the cell culture supernatants were collected and assayed for lactate dehydrogenase (LDH) and IL-6 synthesis.

3.3.4 The effect of molasses samples on phytoheamagglutinin (PHA) stimulated and unstimulated WBCs

Stimulated whole blood cultures contained 10 volumes of blood and 89 volumes of RPMI-1640 medium (Sigma-Aldrich, USA) with PHA (Sigma-Aldrich, USA) in RPMI at a final concentration of 16 µg/ml PHA. For unstimulated whole blood cultures, no additions were made to the diluted blood. Dilution ranges of molasses (Health Connections Wholefoods, SA) in distilled water was dispensed at 3 µl/well in wells of 96-well plates (Nunc-Immuno plate, Serving Life Science, Denmark). PHA stimulated or unstimulated diluted blood (300 µl/well) was added to molasses samples and thereafter incubated at 37 °C for 48 hours. At the end of the incubation period the cell culture supernatants were collected and assayed for IFN-γ and IL-10 synthesis.

3.3.5 The effect of natural and artificial sweeteners on stimulated and unstimulated WBCs

The artificial sweeteners used in this study were categorized into their respective groups based on their primary constituent. The brands Canderel™ and Equal™ mainly contain aspartame, Natreen™ and Sweetex™ comprise of saccharin and Splenda™ and Swheet™ consist of sucralose. Diluted whole blood was stimulated either with or without mitogens (to give a final concentration of 10 ng/ml LPS and 16 μg/ml PHA). Both commercially available natural (molasses, white sugar and brown sugar) and artificial sweeteners (Canderel™, Equal™, Natreen™, Sweetex™, Splenda™ and Swheet™) were diluted in distilled water and used at a physiologically relevant concentration of 10 μg/ml. LPS stimulated and unstimulated blood (500 μl/well) were added to 48-well culture plates (Nunc-Immuno plate, Serving Life Science, Denmark) and incubated at 37 °C in the presence or absence of sweeteners (5 μl/well) for 18 hours. PHA stimulated and unstimulated blood (500 μl/well) were added to 48-well culture plates and incubated at 37 °C in the presence or absence of sweeteners (5 μl/well) for 48 hours. Control wells consisted of stimulated or unstimulated cells cultured in medium alone. All samples were plated in triplicate. At the end of the incubation period, the culture supernatants harvested from the LPS stimulated cultures were assayed for IL-6 and LDH, while the PHA stimulated supernatants were assayed for IFN-γ and IL-10 synthesis.

3.3.6 Determination of lactate dehygrogenase (LDH) release

Lactate dehydrogenase activity in cell culture supernatants was used to determine cytotoxicity of samples. LDH was measured using a cytotoxicity detection kit (Biovision, USA). The kit contained all components required for the assay. Cells

were lysed with cell lysis solution and used to determine total cellular LDH. Cell culture supernatants were collected for the assay and lysed cells were assayed on a 96-well plate (Nunc-Immuno plate, Serving Life Science, Denmark). Reaction mixture (100 µl) was added to each well and incubated for approximately 15 minutes. The absorbance of reaction mixtures were then measured at 492 nm using an ELISA reader.

3.3.7 Cytokine ELISAs

Whole blood culture supernatants were screened for IL-6, as a biomarker of inflammation, IFN-γ as a biomarker of cell mediated immunity and IL-10 as a biomarker for humoral immunity. Cytokine production by whole blood cultures were measured using ELISA kits (eBioscience, USA). The kits contained all reagents required for the assay. Briefly, 96-well plates (Nunc-Immuno plate, Serving Life Science, Denmark) were coated with 100 µl per/well of capturing antibody diluted appropriately in coating buffer and incubated overnight at 37 °C. After 5 washings with wash buffer (autoclaved phosphate buffered saline containing 0.05 % Tween-20), non-specific binding sites were blocked with assay diluent for 1 hour at room temperature. Cell culture supernatants (50 µl) were then added to their respective wells. Recombinant human cytokine standards were also included on each plate. The plate was sealed and incubated at room temperature for 2 hours. After 5 washings, 100 µl of detection antibody (Biotin-conjugated anti-human cytokine) was added to each well. The plate was incubated for 1 hour at room temperature. The plate was washed again for 5 times and the biotineylated sandwich was detected by adding 100 µl of the Avidin-horseradish peroxidase conjugate (HRP) to all wells. The plate was incubated for 30 minutes. After 7 washings the bound peroxidase was monitored by adding 100 µl of the substrate solution (Tetramethlybenzidine solution)

to every well. The plate was incubated for approximately 15 minutes after which the reaction was stopped with the addition of 50 µl stop solution to all wells. Absorbance was read at 450 nm on an ELISA plate reader.

3.3.8 Statistical analysis

All data was statistically analysed via one-way ANOVA using SigmaStat software (Systat Software Inc., USA). All cytokine assays were conducted in triplicate to avoid statistical errors. Results were expressed in terms of the mean ± standard deviation or the mean ± standard error of the mean (SEM).

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3.4 Results

The effect of sugar cane molasses on the immune system

3.4.1 Cytotoxicity of sugar cane molasses

Molasses samples were tested for cytotoxicity using LDH release from whole blood cultures as a biomarker. Upon exposure to toxic compounds, the cells die and due to leaching, release LDH in to the medium. The standard curve of various dilutions of the total cellular LDH shows that there is a linear relationship between absorbance (OD) and percentage toxicity (figure 3.1). The supernatants of both stimulated and unstimulated whole blood cultures incubated with molasses samples, contained similar LDH levels to the control cultures indicating that molasses is not cytotoxic. (Refer to Table 3.1, Appendix 1).
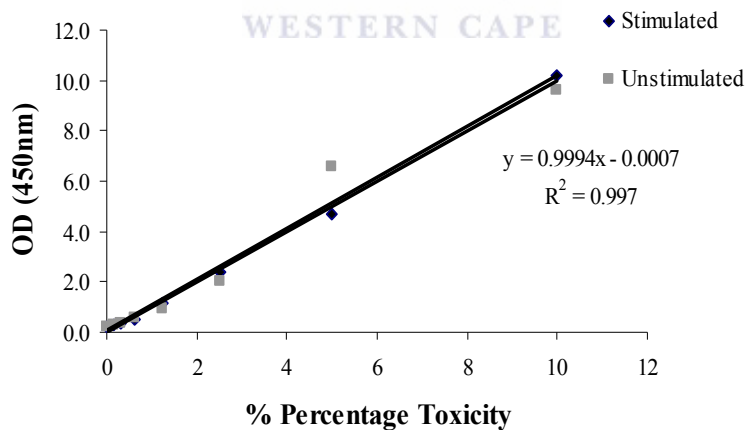
Figure 3.1 A typical standard curve generated for percentage toxicity (%) versus OD at 450 nm. A linear relationship with a good correlation coefficient of 0.997 was observed.

3.4.2 The effect of sugar cane molasses on stimulated and unstimulated WBCs

IL-6 synthesis was used as a biomarker to detect an inflammatory response in both LPS stimulated and unstimulated whole blood cultures. Figure 3.2 illustrates a typical standard curve used to determine IL-6 secretion in WBCs incubated with various sweeteners. A polynomial relationship between OD and IL-6 synthesis (pg/ml), with a correlation coefficient of 0.993 was observed. Upon PHA stimulation of WBCs, the T cell derived cytokines IFN-γ and IL-10 are produced (Hussain et al., 2002). IFN-γ was used as a biomarker for cell mediated immunity and IL-10 as a biomarker to determine the humoral immune response. Figure 3.2 also illustrates the typical standard curves constructed to determine IFN-γ and IL-10 synthesis in WBCs exposed to different sweeteners. A linear progression for both IL-10 and IFN-γ standard curves, with correlation coefficients of 0.985 and 0.998 were observed respectively.
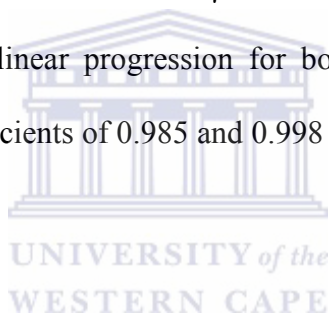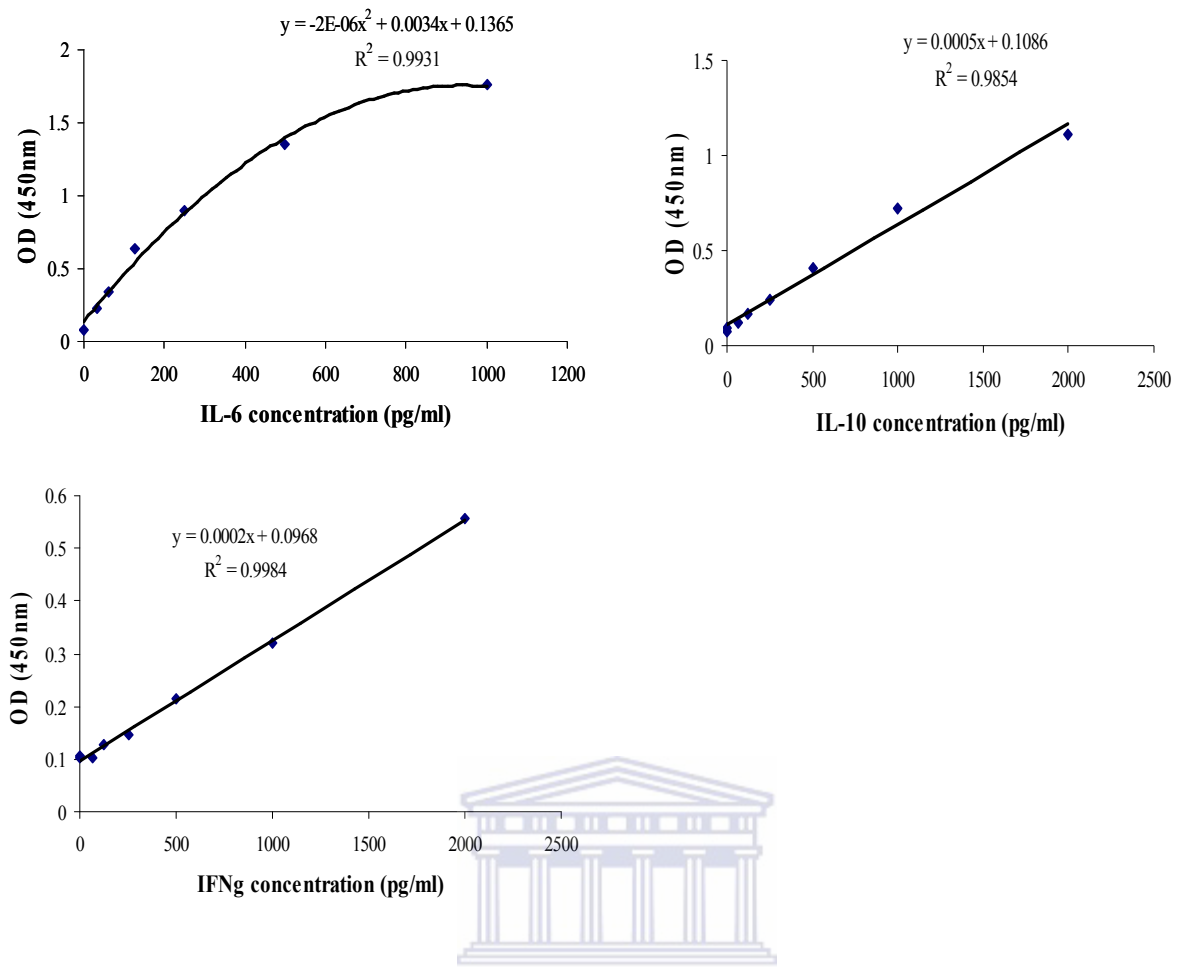
Figure 3.2 Standard curves for IL-6, IFN-γ and IL-10 synthesis (pg/ml) versus OD (450 nm). A polynomial relationship for the IL-6 standard curve with a correlation coefficient of 0.993 was generated. Standard curves obtained for IL-10 and IFN-γ showed a linear progression with correlation coefficients of 0.985 and 0.998, respectively.

3.4.3 The inflammatory activity of molasses samples

Exposure to an immunotoxic sample may produce an elevation or suppression in the synthesis of the pro-inflammatory biomarker, IL-6. The whole blood culture assay for molasses were repeated using four different donors. Results obtained for the donors were similar and figure 3.3 depicts the average obtained for the whole blood cultures of four donors. Molasses has no effect on IL-6 synthesis by stimulated whole blood

cultures (P=0.435). Molasses samples do however have a major effect on IL-6 secretion by unstimulated whole blood cultures. The addition of molasses (12.5–800 µg/ml) samples to unstimulated whole blood cultures resulted in a significantly higher IL-6 secretion compared to the controls (P<0.001). These results indicate that molasses samples stimulate inflammatory activity *in vitro*.
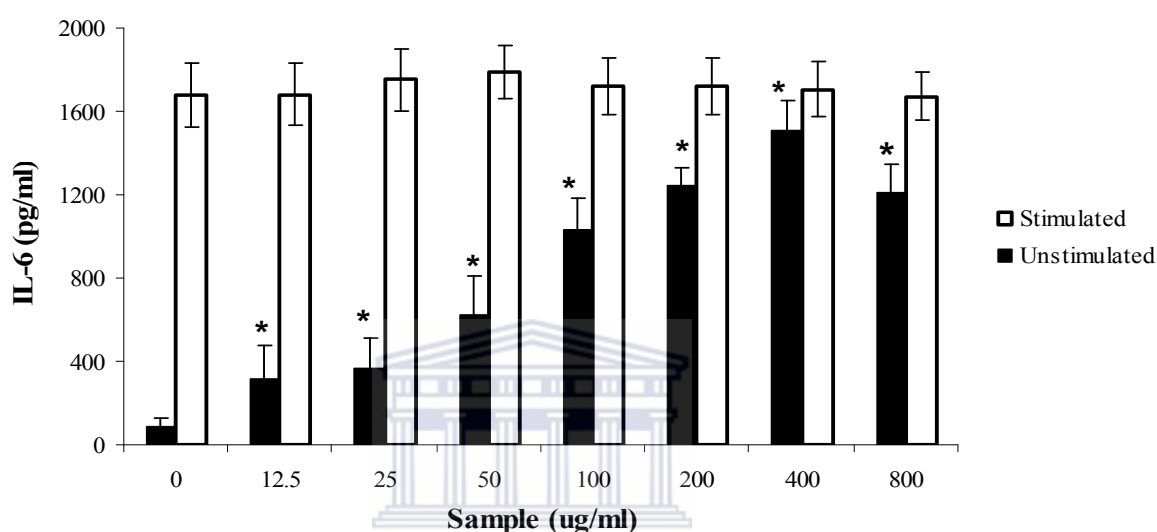
Figure 3.3 Induction of IL-6 (pg/ml) of whole blood cultures *in vitro* by LPS, in the presence of various concentrations of molasses samples and distilled water (control). Each point represents the mean and standard deviation of three replicates (n=12). An asterisk (*) designates significant difference to the control (P<0.001). Unstimulated=■; Stimulated=□.

3.4.4 The effect of molasses on T cell differentiation

IFN-γ directs the differentiation of naïve T-cells (T0) into Th1 cells while IL-10 directs differentiation of Th2 cells (Viveros-Paredes et al., 2005). Exposure to an immunotoxic sample may produce an elevation or depression in the levels of IL-10 or IFN-γ produced. The whole blood culture assay for molasses was repeated using four

different donors. Results obtained for the donors were similar and figure 3.4 depicts the average IL-10 concentrations obtained for whole blood cultures of the four donors. Molasses samples have a major effect on IL-10 secretion by unstimulated whole blood cultures. The addition of molasses (200–800 µg/ml) to unstimulated whole blood cultures resulted in a significantly higher IL-10 secretion compared to the controls (P<0.001). These results indicate that molasses may have an immunostimulatory effect on the differentiation of Th0 cells to Th2 cells that are responsible for synthesising the cytokines required to mount an effective humoral mediated immune response.

Figure 3.4 Induction of IL-10 (pg/ml) of whole blood cultures *in vitro* by PHA, in the presence of various concentrations of molasses samples and distilled water (control). Each point represents the mean and standard deviation of three replicates (n=12). An asterisk (*) designates significant difference to the control (P<0.001). Unstimulated =■; Stimulated=□.

The supernatants of whole blood cultures incubated with molasses samples were screened for IFN-γ synthesis using blood from four donors. Figure 3.5 depicts an average result of all donors, obtained for the whole blood cultures. Molasses has no effect on IFN-γ synthesis of both unstimulated and stimulated whole blood cultures (P>0.05). These results indicate that molasses has no effect on the differentiation of Th0 cells to Th1 cells that functions in synthesising the cytokines required to mount an effective cell mediated immune response against intracellular pathogens (Takatsu and Kariyone, 2003).
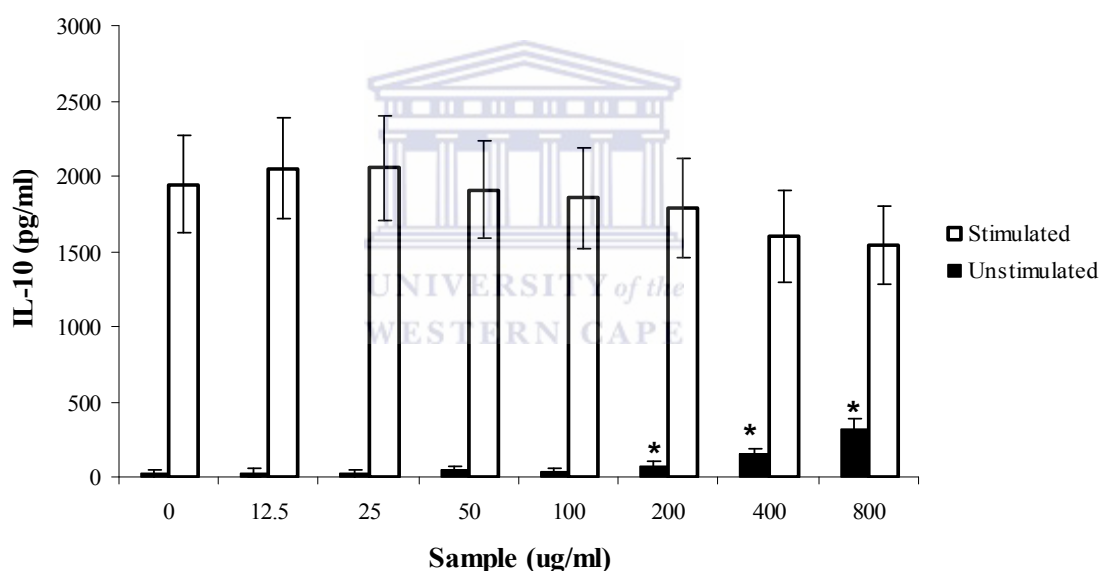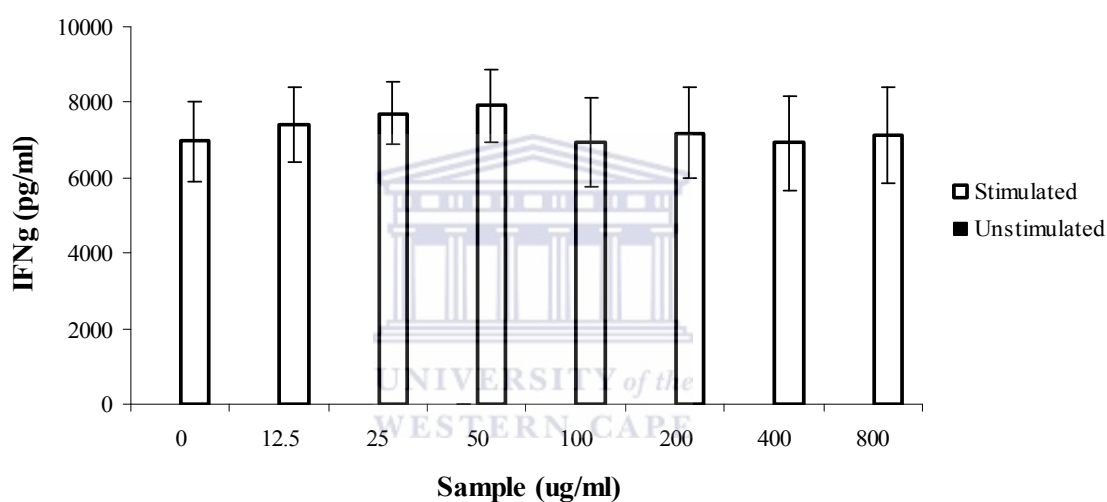

Figure 3.5 Induction of IFN-γ (pg/ml) of whole blood cultures *in vitro* by PHA, in the presence of various concentrations of molasses samples and distilled water (control). Each point represents the mean and standard deviation (n=12). An asterisk (*) designates significant difference to the control (P<0.05). Unstimulated=■; Stimulated=□.

The effect of artificial and natural sweeteners on the immune system

3.4.5 Cytotoxicity of natural and artificial sweeteners

Figure 3.1 presents a typical standard curve generated to determine the percentage toxicity of all samples. Results showed that percentage toxicity obtained for both stimulated and unstimulated cultures incubated with the various sweeteners, were similar to that of the distilled water control. Hence, none of the natural or artificial sweeteners proved to be cytotoxic (Refer to table 3.2, Appendix 1).

3.4.6 The effect of natural and artificial sweeteners on endotoxin stimulated and unstimulated WBCs

Figure 3.6 presents the average IL-6 concentrations obtained for all seven donors under stimulated and unstimulated conditions. Results show that all artificial sweeteners as well as the natural sweetener, sugar cane molasses are significantly different from the control under stimulated conditions ($P<0.001$). In the presence of LPS, all artificial sweeteners suppressed the secretion of IL-6, while sugar cane molasses enhanced the secretion of IL-6 ($P<0.001$) *in vitro*. In the absence of the stimulus LPS, all artificial sweeteners including the natural sweeteners, brown and white sugar showed no effect when compared to the control ($P>0.001$). However, IL-6 levels of unstimulated cultures exposed to sugar cane molasses exceeded that of the control ($P<0.001$).
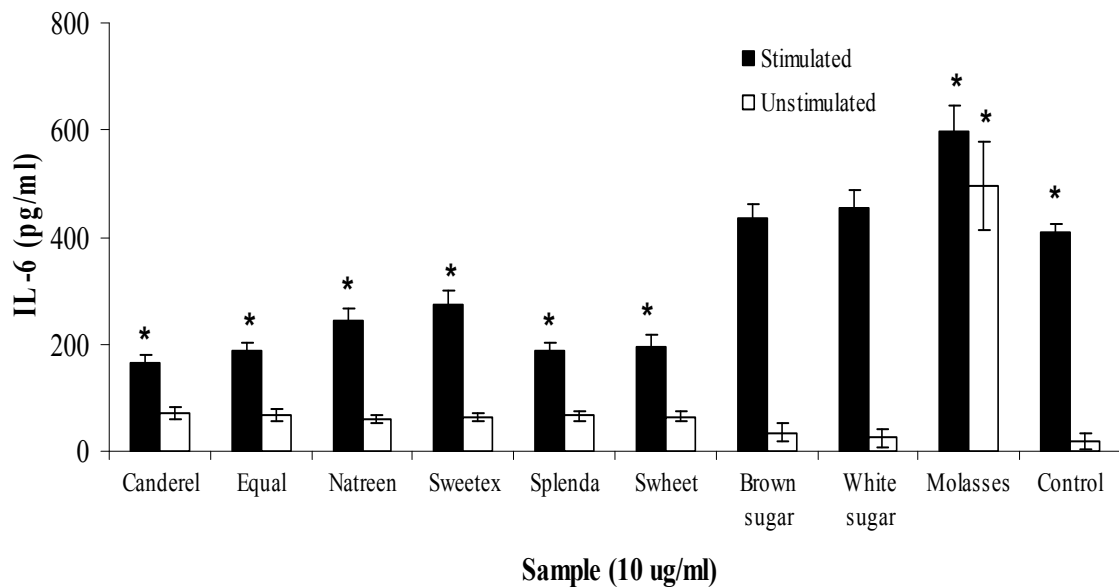
Figure 3.6 The inflammatory activity of artificial and natural sweeteners in LPS stimulated and unstimulated whole blood cultures. Data is expressed as the mean ± standard error of the mean of three replicates (n=21). An asterisk (*) indicates statistical difference to the distilled water control (P<0.001). Stimulated=■; Unstimulated=□.

3.4.7 The effect of natural and artificial sweeteners on PHA stimulated and unstimulated WBCs

Figures 3.7 and 3.8 presents the average IL-10 and IFN-γ levels obtained for all donors under stimulated and unstimulated conditions. Results show that under stimulated conditions, the lowest levels of IL-10 secretion occurred in WBCs incubated with the artificial sweeteners, Splenda™ and Swheet™ (P<0.001). These results reflect that sucralose may have an immunosuppressive effect on the humoral immune response (figure 3.7). Both natural and artificial sweeteners showed no significant difference when compared to the control under unstimulated conditions (P>0.001). The Th1 cytokine, IFN-γ enhances the activation of macrophages, antibody-dependent cellular cytotoxicity as well as the complement pathway. This

form of immune protection functions in defense against intracellular pathogens and any disruption caused to these cells may lead to tissue damage in the host (Langezaal, 2002). The comparison of various sweeteners to the control cultures under both stimulated and normal conditions showed that none of the sweeteners had an effect on IFN-γ synthesis (P>0.05). Hence, none of the sweeteners had an impact on cell-mediated immunity.
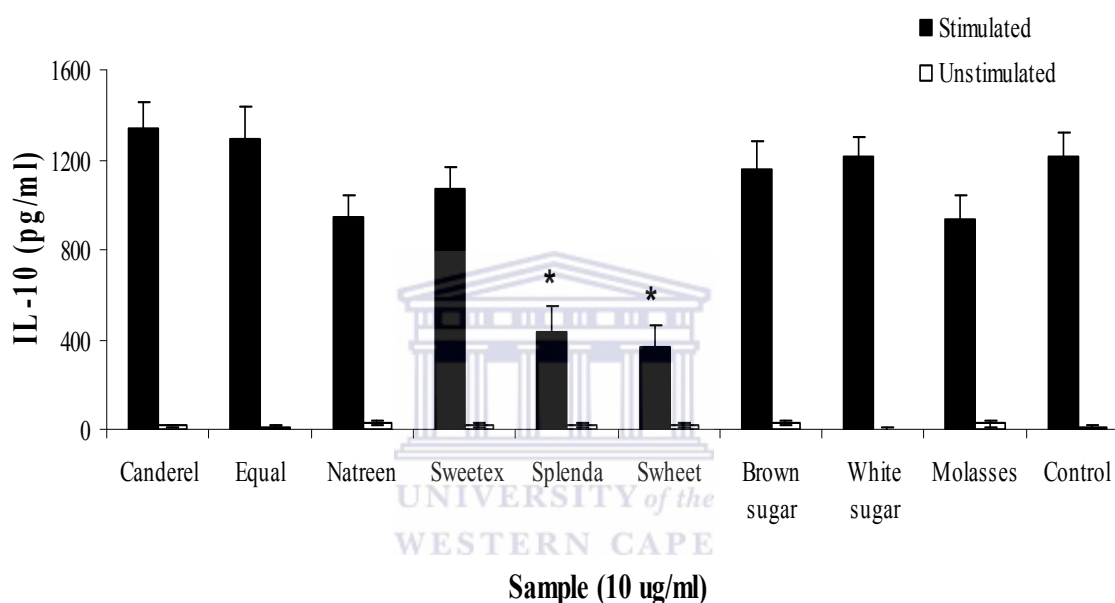
Figure 3.7 IL-10 synthesis by PHA stimulated and unstimulated WBCs incubated with artificial or natural sweeteners. Data is expressed as the mean ± standard error of the mean of three replicates (n=12). An asterisk (*) indicates the statistical difference to the distilled water control (P<0.001). Stimulated=■; Unstimulated=□.
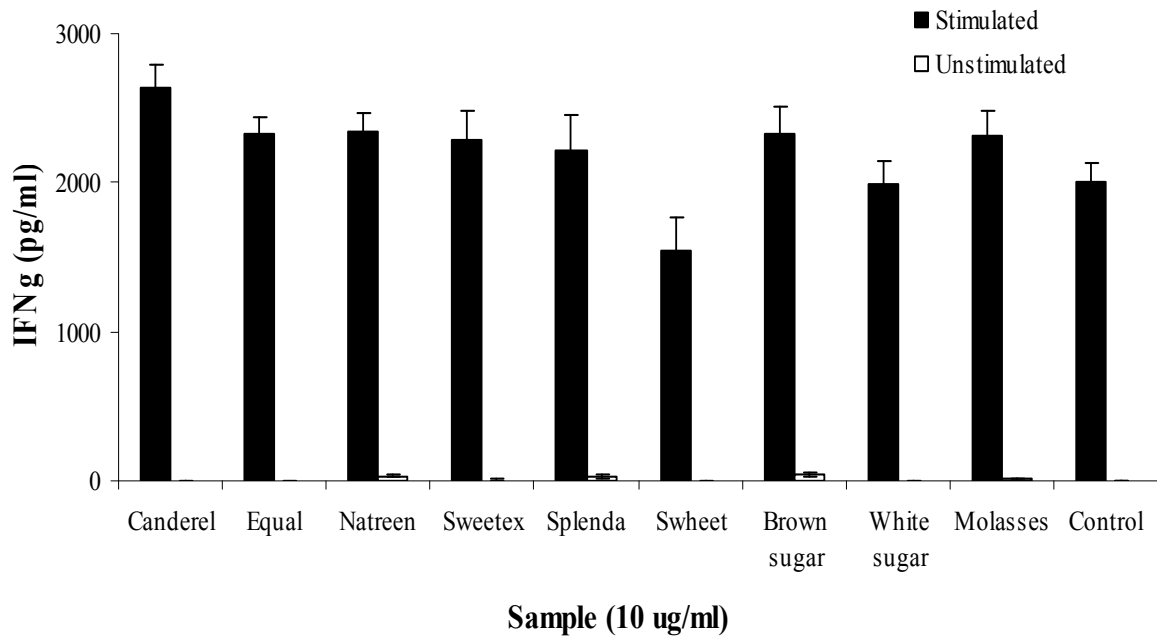
Figure 3.8 IFN-γ synthesis by PHA stimulated and unstimulated WBCs incubated with artificial or natural sweeteners. Data is expressed as the mean ± standard error of the mean of three replicates (n=21). An asterisk (*) indicates the statistical difference to the distilled water control ($P > 0.05$). Stimulated=■; Unstimulated=□.
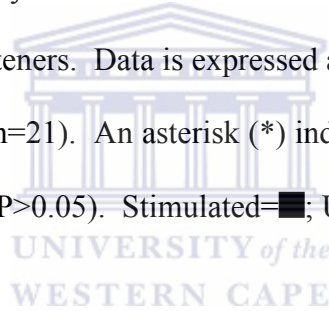
3.5 Discussion

This study shows that certain sweeteners have an impact on cytokine secretion in stimulated or unstimulated whole blood cultures. Whole blood cultures are sensitive to endotoxin stimulation and employ the human fever response to investigate pyrogenic contamination (Langezaal et al., 2001). Tumor necrosis factor-alpha (TNFa), IL-1, IL-6 and IL-8 are inflammatory cytokines that are multi-facetted mediators of the immune system and have various biological functions (Barak et al., 2002).

Results for the first objective of this study show that molasses increases the synthesis of cytokines, IL-6 and IL-10 under unstimulated conditions. IL-6 is a pleiotrophic, inflammatory cytokine that is produced by various cells such as mononuclear phagocytes, fibroblasts and endothelial cells (Abbas and Lichtman, 2001; Barak et al., 2002). IL-6 functions in both cellular and humoral responses. IL-6 also stimulates B cells to induce antibody production and hepatocytes to synthesis acute phase proteins (Kishimoto, 2006). IL-10 is an anti-inflammatory cytokine produced by Th2 cells, which stimulates humoral immunity i.e. B cell activation and maturation resulting in antibody production (Viveros-Paredes et al., 2005; Barak et al., 2002; Storni et al., 2005). The current study shows molasses increases both IL-6 and IL-10 which are necessary requirements for B cells to synthesise antibodies. The data thus indicates that molasses may in fact upregulate humoral immunity.

The increase in the levels of both IL-6 and IL-10 by molasses can be associated with the upregulation of antibody production. Upregulation of antibody synthesis increases the defence against the occurrence of recurrent, extracellular pathogens and their toxins (Roitt et al., 1989). Antibodies are important for opsonization that enhances

phagocytosis and destruction of extracellular pathogens such as Pneumococcus (Abbas and Lichtman, 2001). Therefore, an efficient humoral response will act to eliminate such infective agents.

Studies have shown that numerous herbs have immunomodulatory activity and exhibit immunostimulatory effects in various ways (Tan and Vanitha, 2004; Liou et al., 2002). Immunostimulants elevate specific immune responses by either increasing phagocytosis or the cell mediated or humoral response. IL-6 is a powerful inducer of B cell activation and many herb components such as aloeride (*Aloe vera*), polysaccharides consisting of glucopyranosyl (*Ganoderma lucidum*), angelan (*Angelica gigas*), ginsenosides (*Ginseng*) and gingerols (*Zingiber officinale*) induce IL-6 synthesis and enhance B cell activity. These herbal components are also capable of stimulating the cell mediated immune system (Tan and Vanitha, 2004). In accordance with the above mentioned study, the immunostimulatory synthesis of IL-6 by molasses may therefore be associated with a very efficient humoral response against extracellular pathogens.

The increase in levels of the inflammatory biomarker, IL-6 and anti-inflammatory biomarker, IL-10 also suggests that molasses has the potential to induce both an inflammatory and anti-inflammatory action on the healthy immune system. This is similar to the results of a study conducted on the herbal remedy, Sambucol. Therefore, molasses like Sambucol may have an immunostimulatory effect when administered to patients who suffer from a depressed immune system. These may include patients suffereing from cancer or Acquired Immune Deficiency Syndrome (AIDS) (Barak et al., 2002). Numerous cytostatic drugs such as cyclophoshamide, cyclosporin A (CsA), prednisone and azathioprine cause immunosuppression in

patients. This may unintentionally lead to numerous forms of cancers and an enhanced risk to bacterial infections (Krzystyniak et al., 1995). Molasses may enhance the body's immune defense mechanisms by increasing B cell activity that may augment antibody synthesis and reduce the risk to these types of infections.

Anecdotal evidence suggests that molasses improves health conditions such as rheumatoid arthritis, osteo-arthritis, nervous system dysfunction and various other disorders (Kirschmann et al., 2007). The current study shows that molasses has effects on cytokines regulating the humoral immune system and has both inflammatory and anti-inflammatory potential. As a result, this compound may prove to be beneficial in promoting human health.

Since sugar cane molasses displayed a significant impact on cytokine release *in vitro*, we aimed to further investigate the biological activity of molasses in comparison to various other natural and artificial sweeteners on the immune system. Results show that all artificial sweeteners significantly decreased the levels of IL-6 *in vitro*. This result may be interpreted as the inability to produce an effective inflammatory defense when challenged with the bacterial pathogen endotoxin. On the contrary, sugar cane molasses elevated the levels of IL-6 secretion. Thus, inflammatory activity is increased and an efficient immune response is produced when posed with a threat. The natural sweeteners, brown and white sugar both showed no effect on IL-6 synthesis under stimulated conditions. Normally, infection or injury caused to the body initiates the synthesis of pro-inflammatory cytokines and the elevated levels of these cytokines are essential for recovery (Robles et al., 2005). The increased inflammatory response produced by molasses under unstimulated conditions may therefore initiate a competent immune defense mounted against invading pathogens

on the healthy immune system. However, this form of 'hyperstimulation' may also be associated with hypersensitivity reactions, which are known to adversely affect the health of an individual. The ability of molasses to enhance inflammatory activity *in vitro* is supported by our earlier data (Rahiman and Pool, 2010). As previously stated, the inflammatory potential of molasses may prove to be therapeutic when administered to patients suffering from immunosuppressive illnesses such as cancer and AIDS (Rahiman and Pool, 2010).

Certain infections can cause chronic or persistent inflammation that ultimately ends in pathological effects such as cancer, cardiovascular disease, age-related chronic diseases and Type 2 diabetes (Robles et al., 2005). The level of cytokine synthesis and the balance between inflammatory cytokines versus anti-inflammatory cytokines determines the time-span as well as the end-result of the immune response (Barak et al., 2002). Results indicate that the artificial sweetener, sucralose branded under the names of Splenda™ and Swheet™ significantly reduced the levels of the anti-inflammatory cytokine, IL-10 under stimulated conditions. The decrease of IL-10 levels by sucralose suggests a diminished humoral immune response against extracellular pathogens such as bacteria, fungi and yeast (Twigg, 2005). The cumulative effect of reduced IL-6 and IL-10 synthesis induced by sucralose, further suggests the suppression of antibody synthesis against infective extracellular pathogens.

3.6 Conclusion

This study reveals that sugar cane molasses has an impact on the humoral immune system and has both inflammatory and anti-inflammatory potential. In comparison to other natural and synthetic sweeteners, results also show that sugar cane molasses was the only sweetener to enhance inflammatory activity *in vitro*. Thus, the inflammatory potential of molasses may be favorable in defense against infective pathogens. In addition, results show that artificial sweeteners containing aspartame, saccharin and sucralose appear to inhibit inflammatory activity *in vitro*. Sucralose-containing sweeteners may potentially reduce humoral immunity thus increasing susceptibility of host defense against extracellular pathogens. The above mentioned sweeteners have displayed immunomodulatory effects *in vitro*, which may be associated with potential adverse or beneficial effects on human health. However, further studies are still needed to determine if these sweeteners display similar effects *in vivo*.

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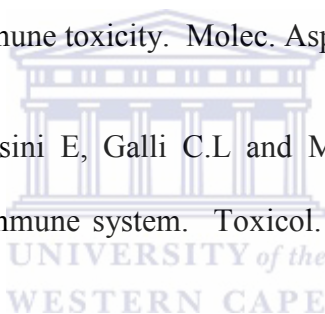
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Chapter 4

The effect of artificial and natural sweeteners on *in vitro* testosterone biosynthesis

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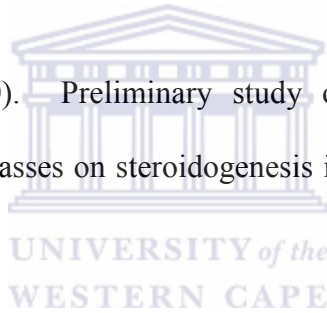
Chapter 4

4.1 Abstract

Artificial and natural sweeteners have been used extensively in the manufacture of food products and have become an important part of the human diet. Reports suggest that naturally and synthetically derived sweeteners may induce severe health problems. The reproductive system is a target for various endocrine modulators. Questions have been raised concerning the effects of everyday food products on reproductive health. This study aims to determine the effects of natural and artificial sweeteners on the process of steroidogenesis using testicular cell cultures. Testes cultures were exposed to both natural (sugar cane molasses, brown sugar and white sugar) and artificial (Canderel™, Equal™, Natreen™, Sweetex™, Splenda™ and Swheet™) sweeteners under LH stimulated and unstimulated conditions. LH stimulation induces testosterone synthesis in these cells. The biomarkers, testosterone, estradiol and lactate dehydogenase activity were measured to evaluate the effects of sweeteners on the male reproductive system. Results revealed that concentrations between 12–50 μg/ml of sugar cane molasses elevate testosterone production in the presence of LH stimulation. Furthermore, when comparing the effects of natural sweeteners to artificial sweeteners, molasses (10 μg/ml) was the only sweetener to exhibit an enhancing effect on testosterone biosynthesis *in vitro*. These findings suggest that perhaps the supplementation of molasses in diets of men suffering with testosterone deficiency may be of beneficial use.

4.2 Introduction

The endocrine system is an important component of the body that functions in the growth, development and reproductive activities of both humans and wildlife (Gunnarsson, 2008). There is growing concern regarding the impact of various substances, such as environmental contaminants and chemicals on the endocrine system and its subsequent effect on the health of humans and animals (Gutendorf and Westendorf, 2001).

An endocrine disruptor may be defined as a compound that alters the function of the endocrine system and may act by imitating natural hormones, blocking hormone receptors as well as initiating change in the metabolism of endogenous hormones (DiDiego et al., 2005). Occupational exposure and diet are sources whereby endocrine disruptors may enter the body of a human or animal, followed by their attachment to specific endogenous receptors (Gunnarsson, 2008; Kumar et al., 2008). This initiates an alteration in the hormonal balance of the body and may induce diseases such as cancer, cryptorchidism, suppression of male reproductive fitness, low sperm counts and delayed onset of puberty (Kumar et al., 2008).

Research suggests that exposure to endocrine disruptors such as environmental contaminants may be linked to the occurrence of various reproductive diseases (Sanderson and Van den Berg, 2003). The male sex hormone, testosterone and female sex hormones progesterone and estradiol play a vital function in ensuring reproductive health (Stocco, 1997). Gonadal steroid hormones are derived from the common precursor cholesterol, a process which requires movement between the mitochondria and smooth endoplasmic reticulum as well as various enzymatic reactions. This process is termed steroidogenesis (Whitehead and Rice, 2006;

Sanderson, 2006). In the male, testosterone functions as an active androgen that is essential to the growth and development of the testes, epididimydes, vas deferens, seminal vesicles and other important parts of the male reproductive tract (United States Environmental Protection Agency (U.S. EPA), 2005). Some endocrine disrupting chemicals affect the production of steroid hormones and/or the enzymes responsible for steroid hormone synthesis. As a result, any change induced by endocrine disruptors on sex hormones, such as testosterone or steroidogenic enzymes may have a significant effect on sexual differentiation and maturity (Whitehead and Rice, 2006).

Over the past half century, an alarming decline in male reproductive health has been reported. A rise in testicular germ cell cancer has been well established internationally, specifically among Caucasians. A four-fold elevation in the incidence of testicular cancer was reported in Denmark, where the cancer registry was first established in 1943. Today, it is believed that a young male has a 1 % likelihood of developing a testicular tumour (Andersson et al., 2008). Other reproductive problems such as cryptorchidism and hypospadia, including testicular cancer are hypothesised to be consequences of environmental changes that have occurred over the years. This is speculated to have influenced the chemical, physical, biological and socio-cultural surroundings of humans (Fernandes et al., 2007).

Majority of foods that are consumed daily contain either natural or synthetic chemicals that may pose a health risk to individuals (Nasreddine and Parent-Massin, 2002). Although the levels of these chemicals present in food may be low, there have nonetheless been safety concerns with regard to their use in food products. Natural contaminants may be present at varying concentrations in food items and numerous

questions remain unanswered as to their effects on consumers. Studies have demonstrated the toxicological characteristics of natural chemicals in both human and animal models. The likelihood of a lifetime of exposure to these compounds as well as their associated adverse effects raises significant health concerns. Therefore, the potential risk of common food products should be investigated (Abbott, 1992).

Reports suggest that both natural and artificial sweeteners may have adverse effects on human health. The consumption of these sweeteners have been linked to numerous and severe health problems. Sugar cane molasses is an example of a natural sweetener that has become a common ingredient in the human diet. Molasses is a viscous substance derived as a by-product of sugar refinement. This residual product has a long history of use in animal feeds with usage ranging from eradication of dust and feed wasteage to becoming an important supply of dietary energy (Curtin, 1983). Anecdotal reports propose that molasses may be used to treat various types of illnesses such as arthritis, ulcers, dermatitits, hair damage, eczema, high blood pressure, constipation, colitis, varicose veins, nerve damage, anaemia and bladder problems in humans (Kirschmann, 2007). However, contradicting evidence suggests that molasses may be associated with various diseases. A study conducted by Rowe et al. (1977) shows that cattle with free access to molasses (3 % urea) and limited amounts of roughage developed a syndrome termed 'molasses toxicity'. It has been demonstrated that molasses toxicity induces effects such as decreased body temperature, fatigue, excessive salivation, rapid breathing and a drunken appearance seen in animals (Pate, 1983). Reports further suggest that blackstrap molasses may have potential endocrine disruptive effects in cattle (Cellar, 2006).

Artificial or synthetically produced sweeteners are considered to be weight-loss products that has made possible the enjoyment for sweet taste with little or no calorie intake (Greenly, 2003; Zygler et al., 2009). Common types of artificial sweeteners include aspartame, acesulfame-K, cyclamate, neotame, sucralose and saccharin. Artificial sweeteners have been emphasised for its beneficial uses by the food industry, however reports suggest that these sweeteners are linked to severe illnesses such as respiratory problems, headaches, cancers, allergies, seizures and immune system dysfunction (Zygler et al., 2009).

It is evident from the previously mentioned reports that both natural and artificial sweeteners may have potential adverse effects on human health. Hence, it is imperative that research is conducted to determine the biological activity of such compounds, which have become an integral part of the human diet.
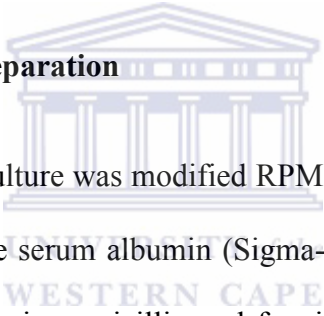
In this study, we aimed to determine the effects of natural and artificial sweeteners on the male reproductive system. The first objective was to investigate the effect of the natural sweetener, sugar cane (*Saccharum officinarium*) molasses on steroidogenesis in testes cultures. Based on the results of this objective, we aimed to further investigate the comparative effects of both natural sweeteners (sugar cane molasses, brown sugar and white sugar) and artificial sweeteners (Canderel™, Equal™, Natreen™, Sweetex™, Splenda™ and Swheet™) on steroidogenesis in testicular cultures. Testosterone and estradiol synthesis were used as biomarkers to determine the potential adverse effects of sweeteners on the male reproductive system.

4.3 Materials and Methods

4.3.1 Animals

Healthy, Balb/C, male mice were purchased from the University of Cape Town's Animal Unit (Cape Town, South Africa) after obtaining animal ethical clearance from the University of the Western Cape. Experiments were conducted in accordance with the guidelines of the institutional animal ethics committee. The mice were kept in a well-ventilated animal house (temperature 20 ± 2 ºC and 12 hour light/ 12 hour dark cycles) in which they had access to normal drinking water and fed standard mouse feed (Medical Research Council, Cape Town, South Africa).

4.3.2 Cell harvesting and preparation

The medium used for tissue culture was modified RPMI-1640 (Sigma-Aldrich, USA), which contained 0.2 % bovine serum albumin (Sigma-Aldrich, USA), 1 % glutamax and 1 % mixture of streptomycin, penicillin and fungizone that was used to prevent contamination (Henceforth, referred to as serum-free medium).

Male mice were sacrificed by cervical dislocation and their testes harvested under sterile conditions. The testes of each of the mice were minced and transferred to a 10 ml tube containing serum free medium. Cell debris was allowed to collect at the bottom of the tube. Following this, the supernatant comprising of cells were pipetted into a new tube. The cells were then centrifuged (Super mini centrifuge, MiniStar Plus, Hangzhou Allsheng Instruments, China) at 1000 x g for approximately 10 minutes. The cell pellet was resuspended in serum-free medium to a final volume of 20 ml. Cells were incubated at 37 ˚C with 5 % CO_2 for 1 hour, after which they were centrifuged and the cell pellet suspended in 20 ml serum free medium. Another

incubation step of 30 minutes at 37 °C with 5 % CO_2 followed. The cells were centrifuged as before and the cell pellet resuspended in 20 ml serum-free medium. Cells were counted using a Neubauer hemacytometer (West Germany) and 10^7 cells per ml were used for tissue culture assays. Cell preparations were then stimulated or not stimulated with 10 mU/ml human luteinizing hormone (LH) (Sigma-Aldrich, USA).

4.3.3 The effect of sugar cane molasses on testes cultures

Dilution ranges of molasses in distilled water or distilled water controls at a volume of 3 µl/well were added to a 96-well culture plate (four replicates per dilution of molasses). The cell suspensions (100 µl/well) were then added to the molasses samples and the plate was incubated at 37 °C and 5 % CO_2 for 4 hours (U.S. EPA, 2005). Following the incubation period, supernatants were collected and assayed for testosterone and estradiol synthesis.
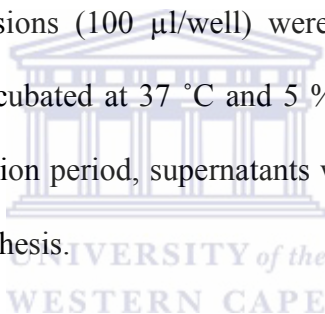
4.3.4 The effect of natural and artificial sweeteners on testes cultures

Both natural (molasses, white sugar and brown sugar) and artificial sweeteners (Canderel™, Equal™, Natreen™, Sweetex™, Splenda™ and Swheet™) used, are commercially available. The artificial sweeteners used in this study may be classified according to their main ingredient. Aspartame-containing products comprise of the brands Canderel™ and Equal™, Natreen™ and Sweetex™ consist of saccharin and Splenda™ and Swheet™ primarily contain sucralose.

Samples were diluted in distilled water and used at a physiologically relevant concentration of 10 µg/ml for all assays. Artificial and natural sweeteners were diluted in serum free medium to a final concentration of 10 µg/ml and added to all

wells at 50 µl per well. Following this, stimulated and unstimulated cells (50 µl) were added to all samples. The plate was then incubated at 37 ˚C with 5 % CO_2 for 4 hours. The control used throughout this study comprised of cells that received distilled water in medium. After the 4 hour incubation period, supernatants were assayed for testosterone and estradiol synthesis.

4.3.5 Testosterone and estradiol ELISAs

Harvested supernatants were screened for both testosterone and estradiol synthesis using commercially available ELISA kits (DRG diagnostics, Germany). All assays were performed as per manufacturer's guidelines. The kit contained all components, including positive controls required to perform the assay. The experiment was done in quadruplicate to avoid statistical errors.

The testosterone ELISA was conducted using 96-well plates pre-coated with mouse-monoclonal anti-testosterone antibody. This assay was standardised using a dilution range of testosterone (0–16 ng/ml) provided with the kit. Collected supernatants and standard samples (25 µl/well) were added to appropriate wells. Enzyme conjugate (testosterone conjugated to horseradish peroxidase) was then dispensed at 200 µl to all wells. This followed an incubation period of 60 minutes at room temperature after which plates were washed three times with wash buffer supplied with the kit. Plates were tapped dry to remove residual droplets and substrate solution (1 x tetramethylbenzidine (TMB)) was then added (200 µl) to all wells for approximately 15 minutes. The reaction was stopped using 100 µl of 0.5 M H_2SO_4 as stop solution. Plates were read at an absorbance of 450 nm.

The estradiol assay was performed on 96-well plates precoated with anti-estradiol antibody. The assay was standardised using a dilution range of estradiol contained within the kit (0–2000 pg/ml). Standard samples and harvested supernatants were dispensed at 25 µl to relevant wells. Following this, 200 µl of estradiol conjugated to horseradish peroxidase was added to all wells and the plates were incubated at room temperature for a duration of 2 hours. Plates were then washed three times with wash buffer supplied within the kit. After plates were tapped dry, 100 µl of warm substrate solution (TMB) were added to all wells for 15 minutes. Stop solution (0.5 M H_2SO_4) was then added at 50 µl per well to stop the enzymatic reaction. Absorbances were read at 450 nm using a spectrophotometer.

4.3.6 Determination of lactate dehydrogenase (LDH) release

Lactate dehydrogenase activity in cell culture supernatants was used to determine cytotoxicity of samples. LDH was measured using a cytotoxicity detection kit (Biovision, USA). The kit contains all components required for the assay and was performed in accordance with the manufacturer's instructions. Briefly, 10 µl of cell culture supernatants were transferred to a 96-well plate (Nunc-Immuno plate, Serving Life Science, Denmark) and 100 µl of cytotoxicity kit reaction mixture was added to each well. The plate was then incubated for 15 minutes at room temperature. The absorbance of reaction mixtures at 492 nm were measured at time zero and after 15-30 minutes using a plate spectrophotometer (Original Multiskan EX, Type 355, Thermo Electron Corporation, China).

4.3.7 Statistical analysis

SigmaStat software (Systat Software Inc., USA) was used for statistical analysis of all samples. All assays were performed in quadruplicate to avoid statistical errors and data was determined using one-way ANOVA and regression analysis. Data is expressed as mean ± standard deviation or mean ± standard error of the mean (SEM).

4.4 Results

4.4.1 The effect of sugar cane molasses on testes cultures

Molasses extracts were tested for cytotoxicity using LDH release as a biomarker. Treatment with a toxic compound initiates the release of the LDH enzyme into culture medium due to cell membrane damage (Fotakis and Timbrell, 2006). The amount of LDH present in culture supernatants were expressed as a percentage of total cellular LDH. A standard curve similar to that presented in figure 3.1 was used to calculate percentage toxicity of all samples. Results showed that none of the molasses extracts resulted in a significant increase in LDH release when compared to controls ($P>0.05$), indicating that molasses is not cytotoxic (Refer to table 4.1, Appendix 1).
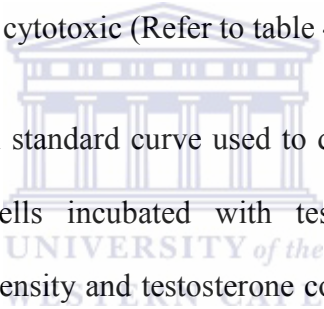
Figure 4.1 illustrates a typical standard curve used to determine the concentration of testosterone produced by cells incubated with test samples. A logarithmic relationship between optical density and testosterone concentration with a correlation coefficient of 0.993 was observed. Molasses addition (12.5–50 µg/ml) to LH stimulated testes cultures increased testosterone production *in vitro* ($P<0.05$) (figure 4.2). This experiment was repeated with testes from three mice and all of these experiments gave similar results. Molasses has no effect on testosterone secretion by unstimulated cultures (figure 4.2). In addition, estradiol was not detected under any of the conditions investigated ($P>0.05$) (Refer to table 4.2, Appendix 1).

The figure shows OD (450 nm) on the y-axis versus Testosterone (ng/ml) on the x-axis (log scale).

$y = -0.5268 \ln(x) + 1.7238$

$R^2 = 0.9937$
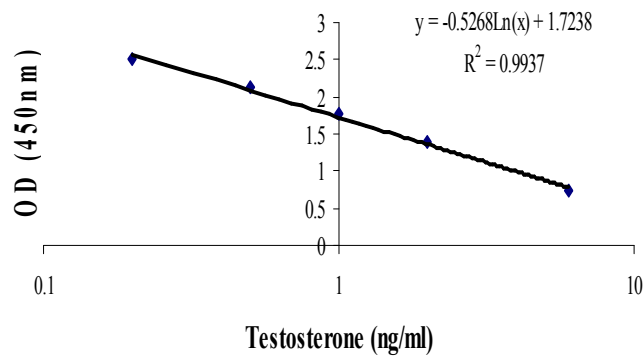
Figure 4.1 A typical standard curve obtained for the testosterone ELISA. A logarithmic relationship between OD and testosterone synthesis with a correlation coefficient of 0.993 was observed.
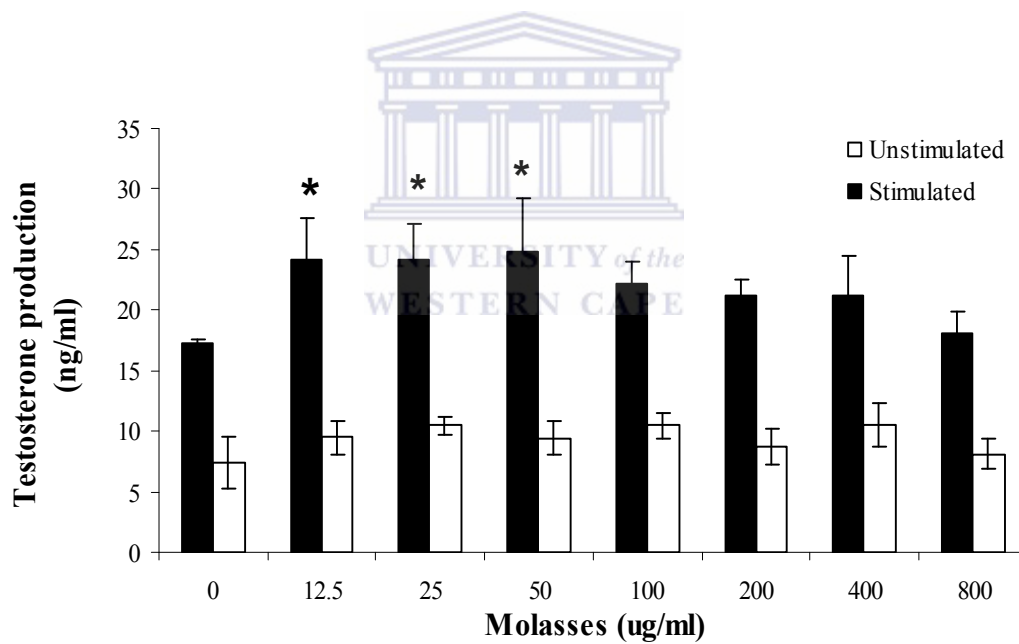
Figure 4.2 The effect of molasses on testosterone production by testes cell cultures (* indicates P<0.05 relative to the distilled water control). Each point is the mean and standard deviation of four replicates (n=12). Stimulated=■ ; Unsimulated=□.

4.4.2 The effects of natural and artificial sweeteners on testes cultures

The supernatants of whole blood cultures incubated with either artificial or natural sweeteners were screened previously to determine potential cytotoxicity. Results revealed that cultures containing sweeteners produced similar levels of LDH when compared to control cultures. Thus, all artificial or natural sweeteners investigated in this study have proven to be non-cytotoxic (Refer to table 3.2, Appendix 1).

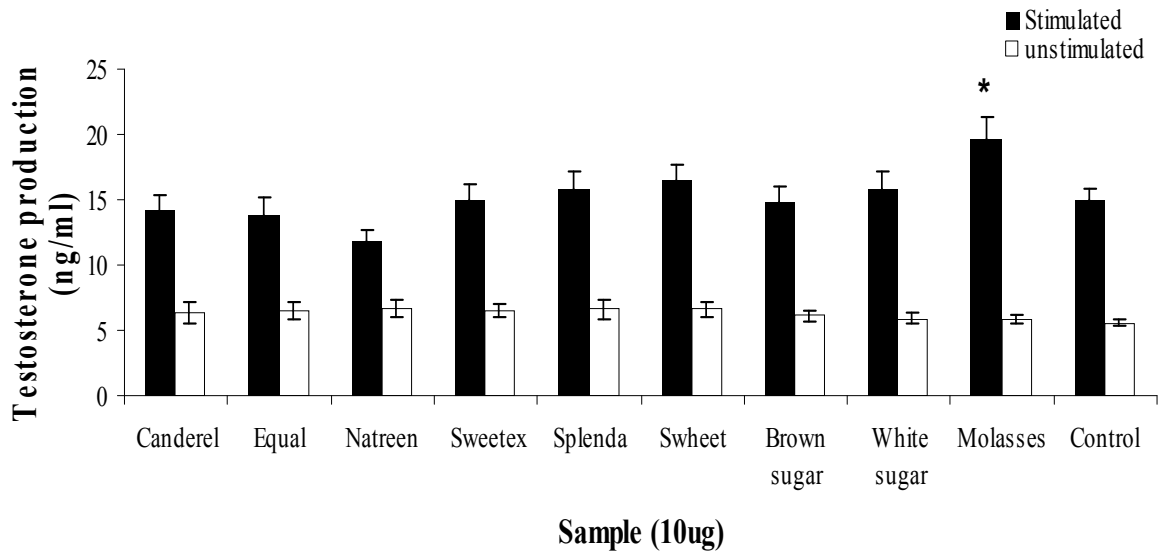Figure 4.3 illustrates the effect of artificial and natural sweeteners on testosterone synthesis in stimulated and unstimulated testicular cultures. This figure presents the data obtained as an average of eleven mice for stimulated cultures and as an average of four mice for unstimulated cultures. Results showed that no significant difference between control cultures and cultures incubated with sweeteners was observed under unstimulated conditions (P>0.05). The addition of artificial sweeteners i.e. Canderel™, Equal™, Natreen™, Sweetex™, Splenda™ and Swheet™ showed no effect on testosterone production in LH stimulated testes cultures (P>0.05). Natural sweeteners namely, brown sugar and white sugar also showed no effect on hormone synthesis under stimulated conditions. However, sugar cane molasses increased LH induced testosterone synthesis *in vitro*, thus demonstrating potential androgenic properties (P<0.05). This result is consistent with earlier findings obtained for this study (Rahiman and Pool, 2010). Testicular cells treated with sugar cane molasses also showed little or no estradiol synthesis under both stimulated and unstimulated conditions (P>0.05). Exposure of testes cultures to both artificial and natural sweeteners showed a similar response for estradiol secretion (Refer to table 4.3, Appendix 1).

Figure 4.3 The effect of artificial and natural sweeteners on testosterone synthesis in testes cell cultures (* indicates $P<0.05$ relative to the distilled water control). Each point is the mean ± standard error of the mean of four replicates, where n=44 for stimulated cultures and n=16 for unstimulated cultures. Stimulated=■; Unsimulated=□.
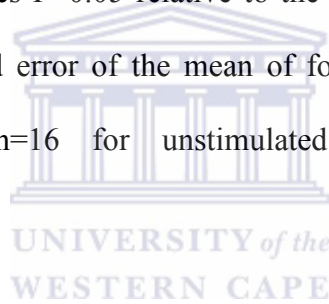
4.5 Discussion

Results show that sugar cane molasses has a significant effect on testosterone secretion *in vitro* and thereby on the process of testicular steroidogensis. Testicular steroidogenesis is essential to the growth and development of the male reproductive tract (Gunnarsson, 2008). Recent research shows that this biosynthethic pathway has become the target of various endocrine disruptors (Sanderson and Van den Berg, 2003). This pathway is modulated by gonadotrophins as well as steroid hormones and key enzymes (Whitehead and Rice, 2006, Sanderson 2006). Therefore, any malfunction that affects these regulatory mechanisms or enzymes, can in turn affect hormone synthesis, which may lead to male reproductive toxicity. The disruption of gonadal steroidogenesis may result in either an increase or decrease of sex hormones or the manufacture of new products. Research has shown that a toxic insult to the reproductive tract may be associated with reduced fertility or sterility, cancer and abnormal sexual and physical growth (U.S. EPA, 2005).

Testosterone is a sex steroid that is required for the growth of the internal and external male reproductive tract (Fisher, 2004). Testosterone functions in maintaining libido, sperm production, muscle and bone mass as well as male hair patterns (Vassan, 2006). Therefore, it is vital that normal levels of testosterone are established in order to maintain reproductive health. This study shows that molasses extracts elevate testosterone production by LH stimulated testicular cells *in vitro* and as a result may therefore be used in the regulation of the above mentioned male biological processes. There appears to be little or no scientific evidence that directly associates molasses with increased testosterone production. On the contrary, reports suggest that molasses is a potential endocrine disruptor (Cellar, 2006). The stimulation of testosterone

production by molasses *in vitro*, may prove to be beneficial as a supplement in diets of men with low testosterone levels. Numerous studies with men ranging from middle to older age have shown an association of decreased testosterone levels with various diseases such as poor memory and cognitive potential, metabolic syndrome, osteoporosis, sarcopenia and Type 2 diabetes (Yeap, 2008). Perhaps the supplementation of molasses in diets of older men may prove to be favourable for future use.

The second objective of this study was to determine the comparative effects of natural and artificial sweeteners on the male reproductive system. Results show that none of the artificial sweeteners investigated had an impact on the male reproductive system. Conversely, the natural sweetener sugar cane molasses demonstrated its stimulatory potential by inducing high levels of testosterone production in LH stimulated cultures. This result supports our preliminary data obtained for the effect of molasses on steroidogenesis in testicular cultures (Rahiman and Pool, 2010). The increase observed in testosterone secretion may be due to the modulatory effect of molasses acting on particular points of the steroidogenic pathway. An upregulation of testosterone synthesis may be associated with an enhancement in the process of steroidogenesis that may be a result of either increased enzyme activity or an elevation of the gonadotophin, LH.

We stated earlier that perhaps the use of sugar cane molasses may prove to be a beneficial supplement in diets of males who suffer with testosterone deficiency (Rahiman and Pool, 2010). Findings for our second objective also suggest that the inclusion of sugar cane molasses as a dietary supplement may augment testosterone production and in doing so may improve reproductive health.

4.6 Conclusion

Data determining the effect of sugar cane molasses on the male reproductive system showed that molasses has the potential to act as a stimulant of testosterone production *in vitro*. The current study also revealed when comparing the effects of natural sweeteners against artificial sweeteners, that molasses was the only sweetener to have an effect on testicular steroidogenesis by enhancing testosterone synthesis. This finding was consistent with earlier results that also reflected an elevation in testosterone secretion by molasses. Thus, sugar cane molasses may possess potential androgenic properties that may be used beneficially to improve reproductive health. However, additional research on artificial and natural sweeteners is required to investigate whether these sweeteners display similar effects *in vivo*.

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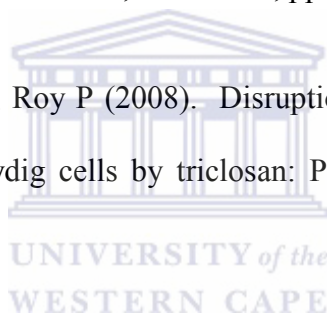
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Chapter 5

The effect of artificial and natural sweeteners on steroidogenesis in ovarian cell cultures

Chapter 5

5.1 Abstract

Various compounds of both natural and synthetic origin have the ability to interfere with the process of ovarian steroidogenesis. The alteration in the synthesis of key steroid hormones may lead to impairment in vital reproductive processes such as ovarian growth and function. This study investigates the effects of artificial and natural sweeteners on the steroidogenic pathway using frog, ovarian cell cultures. Cultured *Xenopus* oocytes were incubated with natural (brown sugar, white sugar, molasses) or artificial (aspartame, saccharin, sucralose) sweeteners in the presence and absence of luteinising hormone. Luteinising hormone stimulates the synthesis of the steroid hormones, testosterone and estradiol. Supernatants of ovarian cultures were then screened for testosterone (T) and estradiol (E_2) synthesis. Results showed that the saccharin-containing sweetener, Natreen™ exhibited potential androgenic properties while the natural sweetener, molasses demonstrated potential estrogenic effects *in vitro* ($P>0.05$). These results were supported by a correlating decrease by Natreen™ or increase by molasses on the E_2/T ratio ($P>0.05$). Aspartame and sucralose branded sweeteners decreased E_2 synthesis, which was confirmed by the decrease observed in the E_2/T ratio ($P>0.05$). The potential androgenic, estrogenic or anti-estrogenic properties of specific sweeteners may have either a harmful or favourable effect on the female reproductive tract.

5.2 Introduction

Reproduction and embryo growth, development and maturation, energy maintenance, homeostasis and behaviour are all vital processes that are highly dependent on normal endocrine function in both humans and animals. The communication of hormones with their respective receptors, at a nuclear and cellular level of different organs and tissues, induce intricate reactions that are components of biological messenger mechanisms. Therefore, a disturbance caused at any level of endocrine signalling may result in injury to the physiology of an entire organism (Caserta et al., 2008).

The female reproductive tract is a complex system that undergoes a process termed ovarian steroidogenesis, which facilitates mechanisms such as folliculogenesis, ovulation and pregnancy. Cytokines, gonadotrophins, steroid hormones as well as some exogenous factors are all active participants in the regulation of ovarian development (Uzumcu and Zachow, 2007). Steroidogenesis occurs mainly in layers of granulosa and thecal cells that envelop the growing follicle containing the ovum. Androgens and progesterone synthesised by theca interna cells, operate as a precursor for estrogen production in granulosa cells. Androgens, namely testosterone and androstenedione are then transported to nearby granulosa cells, where the conversion of these androgens to estradiol takes place. The enzymes, aromatase and 17 beta-HSD types 1 and 2 promotes this process and enhances the conversion of estrone to estradiol. During folliculogenesis, the increased activity of aromatase by luteinising hormone (LH) and follicle stimulating hormone (FSH) progressively elevate estrogen levels. The enhanced regulation of LH receptors and the induction of the positive feedback loop are highly reliant upon estrogen levels during this critical period. This in turn increases the secretion of LH and FSH, which prompts ovulation. The luteal

phase follows, during which the follicle develops into the corpus luteum and primarily manufactures progesterone. Declining LH levels and aromatase expression is associated with lowered estrogen secretion, while an increasing CYP11A and 3B-HSD expression elevates the secretion of progesterone (Sanderson, 2006).

Studies have shown that endocrine disruptors interfere with female reproduction processes by targeting the mechanisms involved in ovarian growth and function. It has been proposed that the estrogenic, anti-estrogenic, androgenic and anti-androgenic potential of endocrine modulators are responsible for the adverse effects seen in the female reproductive system (Uzumcu and Zachow, 2007). Synthetic or natural chemicals have the ability to alter steroidogenic enzyme expression, modify the concentration of steroid hormones, as well as imitate and antagonise endogenous hormone activity (Foster et al., 2004). The synthetic estrogen, diethylstilbestrol (DES) is the most studied example of an estrogenic chemical (Jefferson et al., 2007). During 1938 and 1971, DES was administered to pregnant women as an anti-abortive drug used to avoid pregnancy difficulties and miscarriage. Over the years, animal models have been expanded and enhanced to study the negative effects of DES seen in humans. These models have demonstrated and confirmed that in-utero exposure to DES is associated with the development of clear-cell adenocarcinoma of the vagina and cervix seen in females born to pregnant women who had been on the medication (Rasier et al., 2006). Other harmful effects on the female reproductive system include irregularities in estrous cyclicity and ovulation, reduced fertility and the onset of cancer (Jefferson et al., 2007). Woodruff et al. (2008) reports that the intake of drinking water containing disinfection by-products as well as the consumption of fish contaminated with PCBs and various other toxins have shown to cause alterations in menstrual and ovarian functionality. Exposure to lead and chlorodibromoethane in

drinking water are causal factors of a short menstrual cycle, while other studies have demonstrated that a lengthened menstrual cycle may be the result of exposure to EDCs namely pesticides, TCDD, serum PCBs and industrial chemicals such as ethylene glycol ethers (Woodruff et al., 2008).

Phytoestrogens are a cluster of naturally occurring environmental compounds such as isoflavones and lignans, which are present in food products such as soy (Caserta et al., 2008). Genistein is an example of a phyoestrogen that displays both *in vitro* and *in vivo* endocrine disruptive potential (Uzumcu and Zachow, 2007). Studies have reported that offspring of rats treated perinatally or neonatally with genistein showed signs of early female puberty, an increase in uterine weight, as well as a reduction in prostate weight (Masutomi et al., 2003). Reproductive toxicity due to phytoestrogen consumption has been widely demonstrated in animals over the years. This is evident from reports that suggest subterranean clover (*Trifolium subterraneum*) which contained estrogenic substances, was the causal factor for infertility seen in pasture-grazing sheep (Gunnarsson, 2008). Another study revealed that captive, female cheetahs fed a soy-based diet comprising of high doses of phytoestrogens also showed signs of infertility (Setchell et al., 1987; Jefferson et al., 2007). The above-mentioned evidence suggests that phytoestrogens exist in large enough quantities in the environment that may have a detrimental impact on reproductive function (Jefferson et al., 2007).

Endocrine modulators gain entry into the human body through numerous entry routes, such as consuming contaminated water, ingestion of food, inhalation of contaminated air and skin absorption. The main route of exposure to most chemicals proves to occur through food consumption. For example, more than 90 percent of PCBs

consumed daily are reported to be ingested through food (Stamati and Pitsos, 2001). Exposure to both synthetic and natural compounds is inevitable since they occur ubiquitously in daily life and food products (Gunnarsson, 2008). It is therefore, necessary that we subject our daily food products to toxicity screening to ensure their safe use.

There has been much controversy that concerns the use of sweeteners in the human diet. Contradicting evidence suggests that both artificial and natural sweeteners may have possible beneficial or adverse effects that pose a threat to human health. Few studies have been conducted that investigate the effects of sweeteners on the female reproductive system, which is a common target for endocrine disruptors. Therefore, we aim to determine the effects of both artificial and natural sweeteners on specific steroidogenic biomarkers in order to elucidate potential reproductive toxicity. Exposure to artificial (aspartame, saccharin and sucralose) and natural sweeteners (brown sugar, white sugar and sugar cane molasses) on the female reproductive system was investigated using frog, ovarian cell cultures. The steroidogenic pathway was monitored using testosterone and estradiol as biomarkers.

5.3 Materials and methods

5.3.1 Animals

Sexually mature, female frogs (*Xenopus laevis*) were purchased from an accredited supplier. On arrival, frogs were kept in glass tanks for a time period of 2-3 hours, before they were sacrificed. Frogs were handled with care and as far as reasonably practicable, kept out of distress. Animals were euthanized by submersion in 3-aminobenzoic acid ethyl ester (MS222) until reflexes ceased. The recommended and minimum dosage of MS222 that was used was a 5 g/L solution (Torreilles et al., 2009). The frog was placed in solution and the anaesthetic absorbed via the skin of the frog. This process was conducted by an experienced veterinarian. All procedures were performed in accordance with the guidelines and clearance of the ethics committee at the University of the Western Cape.
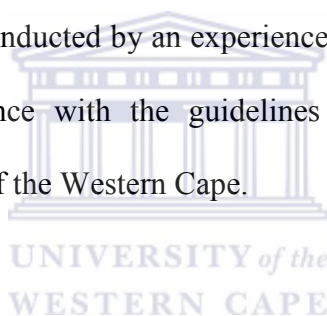
5.3.2 Cell preparation

The ovaries of female frogs were dissected out under sterile conditions. The ovaries were removed and placed in a petri dish containing culture medium (70 % RPMI-1640 with L-glutamine and phenol red (Sigma-Aldrich, USA) and 30 % sterile, distilled water). The culture medium contained a 1 % combination of streptomycin, penicillin and fungizone (Sigma-Aldrich, USA) used to prevent contamination. The ovaries were then carefully sliced into approximately ten oocyte fragments and cultured in 24-well culture plates (Nunc-Immuno plate, Serving Life Science, Denmark).

Natural (sugar cane molasses, white sugar and brown sugar) and artificial sweeteners (Canderel™, Equal™, Natreen™, Sweetex™, Splenda™ and Swheet™) that are commercially available were used for all experiments. The artificial sweeteners used,

may be classified into their relevant groups based on their principal ingredient. The brands Canderel™ and Equal™ consist of aspartame, Natreen™ and Sweetex™ are composed of saccharin and Splenda™ and Swheet™ contain sucralose.

All sweetener samples were reconstituted in distilled water at 10 % (w/v) and stored at 4 $^\circ$C until use. Sweetener samples were diluted in culture medium and used at a physiologically relevant concentration of 10 μg/ml (sugar cane molasses was filtered before use), while control samples consisted of distilled water only. Both sweetener and control samples were added at a volume of 125 μl to the relevant wells containing oocyte fragments. Following this, cell preparations were stimulated or not stimulated with 125 μl of 25 mU/ml human luteinizing hormone (LH) (Sigma-Aldrich, USA) and incubated at 27 $^\circ$C with 5 % CO_2 for approximately 20 hours. At the end of the incubation period, supernatants were screened for protein concentration, testosterone and estradiol synthesis.
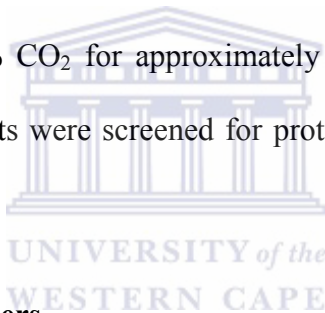
5.3.3 Monitoring of biomarkers

Following the 20 hour incubation period, supernatants were collected and assayed for testosterone and estradiol synthesis. This was conducted using commercially available testosterone and estradiol enzyme linked immunosorbent assay (ELISA) kits (DRG Instruments GmbH, Germany). Assays were performed in accordance with the manufacturer's instructions (refer to 4.3.5). All the reagents required for the ELISAs were supplied with the kit.
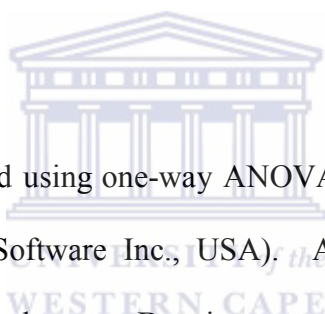
5.3.4 Protein Determination

The protein concentration of all samples was determined using the Bradford Assay (Bradford, 1976). At the end of the 20 hour incubation, culture supernatants were

replaced with 300 μl of a 1 M NaOH solution. This was allowed to gently shake at room temperature for 30 minutes. The supernatant (10 μl) was then collected and dispensed into a 96-well plate (Nunc-Immuno plate, Serving Life Science, Denmark). These sample supernatants were diluted 1:10 with 0.9 % saline. A two-fold serial dilution of bovine serum albumin (BSA) (Sigma-Aldrich, USA) (1.41 mg/ml) in 0.9 % saline was used to produce a standard curve. Protein Assay dye reagent concentrate (Bio-Rad Laboratories, Inc., USA) was diluted 1:4 with distilled water and then added to all wells at 200 μl/well. The plate was incubated for 10 minutes at room temperature and the absorbance read at 620 nm (Original Multiskan EX, Type 355, Thermo Electron Corporation, China).
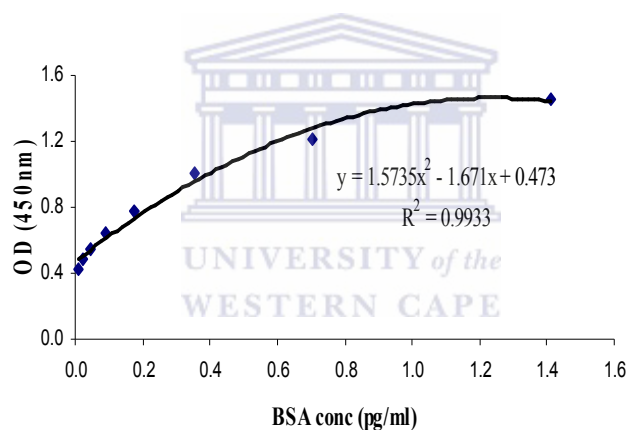
5.3.5 Statistical analysis

Data was statistically analysed using one-way ANOVA and regression analysis with SigmaStat software (Systat Software Inc., USA). All assays were performed in sextuplicate to prevent statistical errors. Data is expressed as mean ± standard error of the mean (SEM) for ten frogs (n=10).

5.4 Results

5.4.1 Protein Determination

Total protein content of oocytes incubated in the presence or absence of sweeteners in each well was determined by producing a standard curve, using BSA as the standard protein. Figure 5.1 illustrates a typical standard curve obtained for assays. A polynomial relationship between protein concentration (pg/ml) and OD (450 nm) with a good correlation of 0.993 was observed. All testosterone and estradiol values were expressed in terms of protein concentration.

The chart shows OD (450 nm) on the y-axis versus BSA conc (pg/ml) on the x-axis, with the fitted equation $y = 1.5735x^2 - 1.671x + 0.473$ and $R^2 = 0.9933$.

Figure 5.1 Standard curve of protein concentration (pg/ml) versus OD at 450 nm. A second order polynomial fit with a correlation coefficient of 0.993 was observed.

5.4.2 The effect of natural and artificial sweeteners on testosterone synthesis in LH stimulated and unstimulated ovarian cell cultures

The concentration of testosterone synthesised by ovarian cells was determined using a standard curve. Figure 5.2 illustrates a typical standard curve showing a logarithmic relationship between testosterone synthesis and absorbance at 450 nm. Table 5.1

presents the average values obtained for testosterone synthesis in ovarian cells exposed to either artificial or natural sweeteners under stimulated and unstimulated conditions. All values were expressed in terms of protein concentration and thereafter as a percentage relative to the distilled water control. Exposure of artificial and natural sweeteners to unstimulated ovarian cells revealed no significant difference when compared to the control (P>0.05). However, in response to the artificial sweetener Natreen™, the stimulatory effect of LH was accompanied by a further elevation of testosterone production (P<0.05).
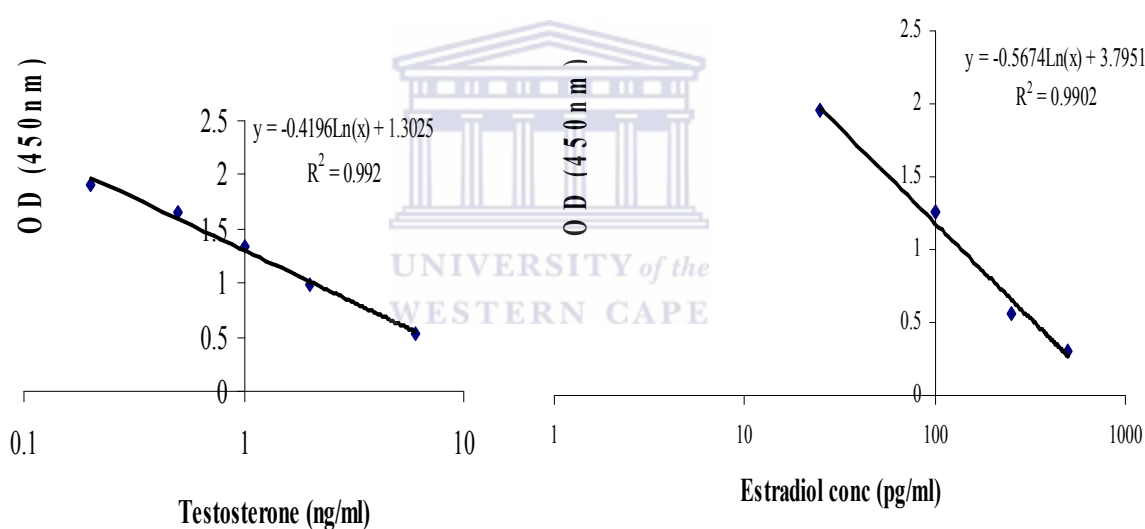
Figure 5.2 Standard curves for testosterone and estradiol concentrations versus OD at 450 nm. Both constructed curves showed a logarithmic relationship between OD and testosterone or estradiol synthesis. Correlation coefficients of 0.992 and 0.990 were observed for testosterone and estradiol synthesis respectively.

Table 5.1 Effect of sweeteners on testosterone synthesis in LH stimulated and unstimulated ovarian cell cultures

	Percentage testosterone (T) (ng/mg protein)			
	Stimulated		Unstimulated	
	Mean	SEM	Mean	SEM
Canderel™	157	25	93	5
Equal™	108	17	137	12
Natreen™	157 *	10	121	9
Sweetex™	105	25	119	15
Splenda™	104	20	132	13
Swheet™	141	13	128	14
Brown sugar	102	12	102	7
White sugar	97	9	98	12
Molasses	109	7	115	6

All values are normalised to protein concentration and expressed as a percentage of the control. For percentage T, values are shown as the mean and standard error of the mean (SEM), n=10 for each treatment. An asterisk (*****) designates the statistical difference of a sweetener sample when compared to the control (P<0.05).

5.4.3 The effect of natural and artificial sweeteners on estradiol synthesis in LH stimulated and unstimulated ovarian cell cultures

The concentration of estradiol synthesised by ovarian cells was determined using the standard curve shown in figure 5.2. A logarithmc relationship between estradiol synthesis and OD, with a correlation coefficient of 0.990 was observed. Table 5.2 indicates the average values obtained for estradiol synthesis by stimulated and unstimulated ovarian cells in the presence or absence of artificial and natural sweeteners. All values were expressed in terms of protein concentration and then as a percentage of the control. Results show that under stimulated conditions eight out of nine sweeteners had no effect on estradiol synthesis when compared to the control

(P>0.05). However, LH stimulated cells incubated in the presence of sugar cane molasses significantly increased estradiol synthesis (P<0.05). In the absence of a stimulus, exposure of cells to the aspartame-containing sweetener, Equal™ showed a reduction in estradiol synthesis (P<0.05). The sucralose branded sweeteners, Splenda™ and Swheet™ also showed a similar effect by significantly suppressing the levels of estradiol production (P<0.05). Conversely, in the absence of LH, molasses once again displayed the potential to induce elevated levels of estradiol synthesis (P<0.05).

Table 5.2 Effect of sweeteners on estradiol synthesis in LH stimulated and unstimulated ovarian cell cultures

Percentage estradiol (E_2) (pg/mg protein)				
	Stimulated		Unstimulated	
	Mean	SEM	Mean	SEM
Canderel™	158	26	96	10
Equal™	131	21	66 *	5
Natreen™	89	3	122	9
Sweetex™	117	22	104	10
Splenda™	99	16	68 *	9
Swheet™	152	22	64 *	7
Brown sugar	108	8	107	6
White sugar	102	7	103	8
Molasses	165 *	10	150 *	9

All values are presented in terms of protein concentration and expressed as a percentage relative to the control. For percentage E_2, values are indicated as the mean and standard error of the mean (SEM), where n=10 for each treatment. An asterisk (*) represents the statistical difference of a sweetener sample in comparison to the control (P<0.05).

5.4.4 The effect of natural and artificial sweeteners on the E_2/T ratio in ovarian cell cultures

Hormone values were expressed in terms of the ratio estradiol (E_2): testosterone (T) to assess testosterone to estradiol conversion. The enzyme aromatase transforms androgens into the corresponding estrogens, which is a critical step in the process of steroidogenesis (Zarn et al., 2003). As a result, this ratio will indirectly give an indication of the impact of test samples on aromatase activity. Data reveals that under stimulated conditions the artificial sweetener, Natreen™ shows an inhibitory effect on the E_2/T ratio ($P<0.05$), while the natural sweetener, molasses significantly increases the E_2/T ratio ($P<0.05$) (Table 5.3). Sugar cane molasses displays a similar effect in the absence of LH by inducing an elevation in the E_2/T ratio ($P<0.05$). The aspartame branded sweetener, Equal™ demonstrates the ability to reduce the E_2/T ratio and may therefore inhibit aromatase activity under unstimulated conditions ($P<0.05$). Splenda™ and Swheet™ also demonstrated a significant inhibitory effect on the E_2/T ratio in the absence of a stimulus ($P<0.05$). Some artificial (Canderel™ and Sweetex™) and natural sweeteners (brown sugar and white sugar) had minimal or no significant effect on testosterone to estradiol conversion under stimulated or unstimulated conditions ($P>0.05$). Exposure of a compound that provokes either an increase or decrease in T to E_2, suggests that the normal conversion of androgen (T) to estrogen (E_2) is affected and as a result may have potential adverse effects on the process of steroidogenesis.

Table 5.3 Effect of sweeteners on the E_2/T ratio in LH stimulated and unstimulated ovarian cell cultures

	Ratio of E_2 vs T			
	Stimulated		Unstimulated	
	Mean	SEM	Mean	SEM
Canderel™	1.062	0.079	1.003	0.06
Equal™	1.234	0.132	0.510 *	0.05
Natreen™	0.558 *	0.052	1.013	0.05
Sweetex™	1.238	0.091	0.930	0.08
Splenda™	0.995	0.049	0.526 *	0.07
Swheet™	1.128	0.177	0.535 *	0.07
Brown sugar	1.123	0.101	1.068	0.06
White sugar	1.080	0.054	1.119	0.08
Molasses	1.580 *	0.153	1.310 *	0.07

All values are expressed in terms of ratio estradiol (E_2): testosterone (T). Data is presented as the mean ± standard error of the mean (SEM), where n=10 for each treatment. An asterisk (*****) designates the statistical difference of a sweetener sample when compared to the control ($P<0.05$).
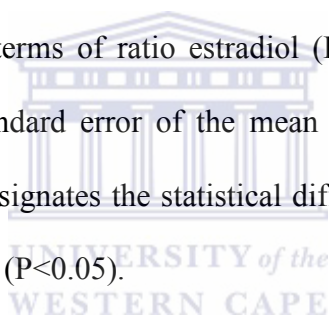
5.5 Discussion

Results of this study show that certain sweeteners have the ability to alter levels of steroid hormones *in vitro*. Steroid hormones are synthesised in vast quantities by the ovary and are essential for the maintenance of follicular and oocyte development, pregnancy, as well as in feedback signalling to the hypothalamus. Interference caused by a compound that alters the androgen-estrogen balance leads to the malfunction of these reproductive processes, impairment in the differentiation of sexual phenotype at the stage of ontogeny and may even incite the development of hormone-dependent cancers (Lenie and Smith, 2009; Zarn et al., 2003). Therefore, it is neccessary that everyday food products are screened for potential adverse effects on organ systems. In this study, the effects of artificial and natural sweeteners on the female reproductive system were monitored by using the steroid hormones estradiol and testosterone as biomarkers. The ratio of E_2 vs. T provides additional information on the potential impact of sweeteners on the process of androgen to estrogen conversion mediated by amongst others, aromatase.

Results show that under stimulated conditions, the artificial sweetener, Natreen™ increased testosterone production when compared to the control. The decrease in the E_2/T ratio by Natreen™ further suggests that the conversion of testosterone to estradiol was reduced. Thus, the increased levels of testosterone in comparison to estradiol synthesis, suggests that Natreen™ may have potential androgenic properties. The hypersecretion of androgens have been associated with various adverse effects on female reproductive health. The role of androgens in pathophysiological functions in females is particularly significant, since they relate to various hyperandrogenic disorders (Foecking et al., 2008). Common reproductive and metabolic diseases in

women are characterised by hyperandrogenemia. Ovarian hyperandrogenism may lead to the development of disorders such as polycystic ovarian syndrome (PCOS), which is frequently accompanied by effects of anovulatory infertility, hirsutism, oligo- or amenorrhea, polycystic ovaries, obesity, insulin resistance as well as hyperinsulinemia (Foecking et al., 2008; Franks et al., 1999). Therefore, the hypersecretion of testosterone synthesis by the saccharin branded sweetener, Natreen™ may induce adverse effects on ovarian steroidogenesis that could potentially lead to the onset of the above mentioned disorders.

The aspartame branded sweetener, Equal™ and the sucralose-containing sweeteners, Splenda™ and Swheet™ significantly reduced estradiol synthesis under normal conditions. These artificial sweeteners also showed an inhibition in the conversion of testosterone to estradiol synthesis (decrease observed in E_2/T ratio), which further confirms low levels of estradiol production. Estrogens are key steroid hormones that are involved in crucial reproductive phases of a female's life. At puberty, estrogens are responsible for the growth and maturation of the endometrium, myometrim and breast stroma, while during adulthood estrogens function in maintaining the menstrual cycle and female secondary sexual characteristics (Greenstein and Wood, 2006). Hence, any alterations in the levels of estrogen synthesis induced by a compound may impair the regulation of these reproductive processes. Di (2-ethylhexyl) phthalate (DEHP) is an example of a reproductive toxicant that has been reported to reduce estradiol levels. In an *in vivo* study conducted with adult cycling rats, DEHP not only decreased estradiol levels but also prolonged estrous cycles and caused an inhibition of ovulation. No ovulation resulted in the absence of the corpora lutea which ended in follicles becoming cystic (Lovekamp-Swan and Davis, 2003).

The synthesis of low estradiol levels by the artificial sweeteners i.e. Equal™, Splenda™ and Swheet™ demonstrated *in vitro*, suggests that these sweeteners may have an adverse impact on steroidogenesis that could result in the inhibition of reproductive processes such as menstruation.

Sugar cane molasses was the only natural sweetener that showed a stimulatory effect on estradiol synthesis under both LH stimulated and unstimulated conditions. The high levels of estradiol production were confirmed by the increase observed in the E_2/T ratio. This result suggests that sugar cane molasses may possess potential estrogenic properties. Estrogens have various beneficial effects, however studies have shown that exposure to elevated levels of this hormone may lead to acute and chronic health disorders in both wildlife and humans (Daston et al., 1997). Estrogenic compounds namely Bisphenol A (BPA) (plasticizer), resveratrol (phytoestrogen) and zearalenone (mycotoxin) alter hormone regulation of the menstrual cycle and in doing so have all shown the ability to prolong the estrous cycle of mice. Genistein is another example of a phytoestrogen that induces lengthened and irregular cycles in adult animals (Crain et al., 2008). Studies have reported that while genistein may possess some beneficial characteristics such as the ability to prevent carcinogen-induced mammary gland cancer in prepubertal rats, it also has the potential to induce uterine adenocarcinoma in mice in later life stages (Jefferson et al., 2007). In humans, an increase in the estrogen to androgen ratio has been reported to be a causal factor of precocious puberty and has been hypothesised to be induced by environmental estrogens (McLachlan et al., 2006). Evidence also suggests that estrogens have been linked to numerous diseases such as tumor development and endometriosis. Endometriosis is a gynaecological disorder that is hormone dependent and has been reported as one of the leading causes of infertility among women (Caserta et al.,

2008). Hence, the potential estrogenic properties of molasses may be associated with an adverse impact on female reproductive health. On the other hand, estrogens have been used as an effective form of therapy for estrogen deficient disorders such as menopause. Estrogen replacement therapy (ERT) has proved to be beneficial in treating symptoms of hot flushes, osteoporosis and cardiovascular disease. Epidemiological studies also report that females who have ever used ERT have shown a reduced risk of developing Alzheimer's disease (Brinton et al., 2000). Thus, our findings suggest that sugar cane molasses may potentially be used as a supplement in the diets of estrogen-deficient women to stimulate estrogen synthesis and ultimately improve female reproductive health.

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5.6 Conclusion

An alteration in the levels of steroid hormones suggests that certain sweeteners have an impact on the process of ovarian steroidogenesis. This study has shown that artificial sweeteners such as the saccharin-containing sweetener, Natreen™ has potential androgenic properties, while the natural sweetener, sugar cane molasses displayed potential estrogenic effects *in vitro*. The potential androgenic and estrogenic properties of these compounds may be associated with either an adverse or beneficial impact on reproductive health. Further *in vivo* studies are warranted to validate the *in vitro* effects observed for these sweeteners.

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5.7 References

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Chapter 6

The effect of sugar cane molasses on physiological

systems using *in vitro* and *in vivo* methods

Chapter 6

6.1 Abstract

This study investigated the effects of sugar cane molasses on various physiological systems using *in vivo* and *in vitro* methods. Molasses was adminstered orally to Balb/C, male mice for a period of 2 months. Animals were randomly assigned into either a treatment or control group. General physiological changes, body weight and molasses intake of all animals were monitored. At the end of the exposure period, collected blood samples were evaluated for potential toxicity using plasma biomarkers and liver enzyme activity. Immunised treated and untreated mice were evaluated for antibody titre to determine the effect of molasses on the immune response. To investigate the impact of molasses on testicular steroidogenesis, testes from both treated and control groups were harvested, cultured and assayed for testosterone synthesis. Results showed that fluid intake by molasses-treated animals was significantly increased and these animals showed symptoms of loose faeces. Molasses had no significant effect on body weight, serum biomarkers or liver enzyme activity ($P>0.05$). IgG anti-antigen levels were significantly suppressed in molasses-treated groups ($P<0.05$). This finding suggests that molasses adversely affects the humoral immune response. Animals subjected to molasses exposure also exhibited elevated levels of testosterone synthesis ($P<0.05$). Consistent with previous findings, this result further promotes the use of molasses as a supplement to increase testosterone levels.

6.2 Introduction

Sugar cane molasses, also referred to as the final effluent of sugar refinement is a dense, darkly coloured substance teeming in minerals (Wang et al., 2011; Reyed and El-Diwany, 2008). Molasses comprises mostly of sugars (approximately 46 % w/w), non-sugar organic materials (eg. phenolic compounds which are substances derived form the sugar cane plant) as well as other compounds synthesised during the manufacturing process (eg. melanoidins, which are end products of Maillard reactions) (Guimarães et al., 2007).

Traditionally, molasses has been used as an alternate sweetener to sugar and included as a common ingredient in various food products (Grandics, 2003, Reyed and El-Diwany, 2008). Worldwide, molasses is primarily used as feed for livestock as it enhances microbial growth in the rumen of animals that promotes the digestion of fibre and non-protein nitrogen (Reyed and El-Diwany, 2008). In addition, molasses has been widely advertised for its therapeutic properties believed to be a result of its rich mineral content (Wang et al., 2011). However, little or no scientific evidence exists to corroborate the suggested health benefits of this substance (Saska and Chou, 2002). Nagai et al. (2001) and Koge et al. (2002) have reported physiological actions of different sugar cane extracts. Their results suggest such sugar cane extracts increase the defence against bacterial and viral infections, enhance immune reactions and possess hepatoprotective and antioxidant properties (Saska and Chou, 2002). Guimarães et al. (2007) states that sugar cane components that display the above-mentioned characteristics are often contained in the resultant by-product, molasses (Guimarães et al., 2007). As a result, the biological activity of molasses should be investigated further.

In contrast to the proposed health benefits of molasses, research has shown that molasses has the potential to induce harmful effects in livestock (Cellar, 2006). The supplementation of molasses in the diet of livestock has been reported to cause diseases such as molasses toxicity, urea toxicity and bloat (Preston et al., 1986). Masgoret et al. (2009) also reported the ability of molasses to induce endocrine disruptive effects *in vitro*. However, in a follow-up*, in vivo* study these effects could not be replicated in Holstein bull calves (Masgoret et al., 2009). Nevertheless, these reports suggest that molasses may be a potential risk factor in the development of animal and human disease and should therefore be screened for potential toxicity. The aim of this study was to determine the impact of molasses on liver function, the immune system and steroid synthesis of male mice treated *in vivo*.

The immune system plays an essential part of protecting healthy tissue against infectious pathogens and disease (Kubena and McMurray, 1996). The immune system is a common target of endocrine modulators and research has shown that exposure to certain compounds (synthetic and natural compounds) may alter the immune response leading to various forms of toxicity (De Jong and Van Loveren, 2007, Krzystyniak et al., 1995). Results showed that molasses modulates the activity of certain immune pathways *in vitro* (Rahiman and Pool, 2010a). Molasses stimulated interleukin-6 (IL-6) and interleukin-10 (IL-10) secretion by whole blood cultures indicating effects on inflammation and humoral immunity respectively. The enhanced humoral response produced by molasses may be associated with increased antibody production that protects against invasion by extracellular pathogens. However, the effects of molasses on inflammation suggest that molasses has the ability to provoke the onset of hypersenstivity reactions (Rahiman and Pool, 2010a).

Reproduction is a crucial stage of development and is required for species survival. The impact of certain toxicants on the reproductive system has lead to adverse effects such as abortions, congenital deformities and infertility that may have a detrimental impact for both the human and animal species (Evans, 2007). Food intake is the most common route of entry for various endocrine modulators (Stamati and Pitsos, 2001). It is therefore important that both manufacturers and consumers are aware of the adverse reproductive effects that may be associated with commonly used food products. Previous studies investigated the effects of commercially, available molasses on the male reproductive system by monitoring steroidogenesis in testicular cell cultures *in vitro* (Rahiman and Pool, 2010b). Specific biomarkers of steroidogenesis that were monitored included testosterone and estradiol synthesis. Results showed that molasses extracts stimulate the production of testosterone *in vitro*. This finding suggests that sugar cane molasses may aid in the upregulation of testosterone synthesis, thereby improving male reproductive health (Rahiman and Pool, 2010b).

The liver is an intricate organ system that functions in the metabolism of carbohydrates, fat and proteins (Giannini et al., 2005). This organ system also plays a major role in excretory and secretory functions in the human body (Aktay et al., 2000). The complexity of the liver has made it a target for various toxicants that results in pathological effects (Ncibi et al., 2008). The changes in levels of liver enzymes are reliable indicators of liver injury (Giannini et al., 2005). The increased levels of the intracellular liver enzymes namely aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma are indicative of injury caused to parenchymal liver cells. The enzyme, alkaline phosphatase (ALP) originates primarily in biliary epithelium and the bile canalicular section of liver cells.

Therefore, an increase in plasma ALP levels may be associated with internal or external hepatic biliary blockage as a result of injury induced on hepatocytes (Field et al., 2008).

6.3 Materials and methods

6.3.1 Preparation of sample for dosing regimen

Range finding studies were initially conducted to determine the physiologically relevant dosage of sugar cane molasses that induced an effect *in vitro* (Rahiman and Pool, 2010a; Rahiman and Pool, 2010b). A dosage that ensures animal survival and that are without significant toxicity or distress to the animals was selected. For this study, commercially available molasses (Health Connections Wholefoods, South Africa) was used at a physiologically relevant concentration of 0.057 g molasses/ml of drinking water for all treated animals.

6.3.2 Animals

All experimental procedures as well as the handling and care of animals were conducted in accordance with guidelines of the ethics committee at the University of the Western Cape. Pathogen-free, Balb/C, male mice weighing approximately 24-30 g were purchased form the University of Cape Town's Animal Unit (Cape Town, South Africa). Animals were housed in plastic cages and kept in a well-ventilated room with a photoperiod of 12 hours and a temperature of 20 ± 2 ºC. Standard mouse feed was always made readily available to all animals. For this study, animals were assigned into two treatment and two control groups comprising of six animals each. Animals were treated *in vivo* for the duration of two months. The treatment and control groups were classified as follows:

- Treatment group one received 0.057 g molasses/ml of drinking water and was monitored for potential adverse effects on the male reproductive system.

- Treatment group two received 0.057 g molasses/ml of drinking water and was immunised two weeks prior to the completion of the two-month feeding period. These animals were monitored for potential adverse effects on the immune system.

- Control group one received normal drinking water and was monitored for potential adverse effects on the male reproductive system.

- Control group two received normal drinking water and was immunised two weeks prior to the completion of the two-month feeding period. These animals were monitored for potential adverse effects on the immune system.

6.3.3 General observations

The molasses mixture was made accessible in calibrated drinking tubes similar to that described by Bachmanov et al. (2002). Consumption of molasses was monitored daily and body weights of animals were recorded every 10 days for the entire exposure period. General physiological changes were also monitored.

6.3.4 The effect of sugar cane molasses on the immune system

Two weeks prior to completion of the exposure period molasses treated and untreated mice were immunised according to the National Institute of Health protocol (2007). *Xenopus* plasma was used as an antigen due to its immunogenicity. The antigen for immunisation was prepared by mixing 50 µl *Xenopus laevis* plasma with 1450 µl of saline (0.9 % NaCl). This was emulsified with an equal volume (1500 µl) of complete Freund's adjuvant. Animals were immunised intraperitoneally (i.p.) with 200 µl of the emulsion. Blood samples (10 µl) for monitoring of the immune response were collected from the tail vein 2 weeks after immunisation. Blood samples were centrifuged at 1500 x g and the serum separated and stored at 4 ºC until

use. The serum was screened for antibody titre (Immunoglobulin-γ (IgG)) using an optimised enzyme linked immunosorbent assay (ELISA).

ELISA to determine IgG titre

Flat bottom 96-well plates (Nunc, Serving Life Science, Denmark) were coated with 50 µl per/well of antigen diluted in 0.9 % saline (1:1000) and incubated overnight at 37 ºC. After this, non-specific binding sites were blocked with 1 % BSA in saline (200 µl/well) for 1 hour at room temperature. At the end of this incubation period, the plate was washed four times with wash buffer (phosphate buffered saline containing 0.05 % Tween-20). A doubling dilution of serum samples in 1 % BSA in saline (ranging from 1:1000 to 1:64000) as well as a control sample (1 % BSA in saline only) were added to respective wells at 50 µl per/well. The plate was sealed and incubated at room temperature for 2 hours. After four washings, 50 µl of rabbit-anti-mouse-IgG horseradish peroxidase conjugate (1:5000 in 0.1 % BSA saline) was added to each well and incubated for 1 hour at room temperature. A final washing step of seven washes followed and 50 µl of warm substrate solution (Tetramethybenzidine solution) was added to all wells. The plate was incubated for 15 minutes at room temperature. The reaction was then stopped with the addition of 50 µl stop solution (1M H_2SO_4) and the absorbance read at 450 nm using a spectrophotometer (Original Multiskan EX, Type 355, Thermo Electron Corporation, China).

6.3.5 The effect of molasses on serum biomarkers

Blood collected by means of tail bleeds of mice were dispensed into heparinised capillary tubes (Lasec, SA). All samples were evaluated immediately for haematocrit. Heparinised capillary tubes containing blood samples were sealed with wax (Vitrix,

Denmark) at one end and centrifuged for 3 minutes at 10,000 rpm in a micro-haematocrit centrifuge (Hawskey, England). The percentage of red cells by volume was then recorded using a micro-haematocrit reader (Hawskey, England). The plasma separated by centrifugation was collected and used to assess lactate dehydrogenase activity (LDH) and protein concentration. Liver injury was also monitored by measuring the liver enzymes ALP, AST and ALT in plasma samples.

Lactate dehydrogenase activity (LDH assay)

LDH activity in blood plasma was measured using a chromogenic cytotoxicity detection kit (Biovision, USA). In brief, plasma samples (5 µl) were dispensed into respective wells on a 96-well plate (Nunc, Serving Life Science, Denmark). LDH-reaction mixture (50 µl) was added to all wells and incubated for approximately 15 minutes at room temperature. Absorbance readings were then recorded at 492 nm using a plate reader (Original Multiskan EX, Type 355, Thermo Electron Corporation, China).

Protein Determination

The protein concentration of all samples was determined according to the procedure described by Bradford (1976). Bovine serum albumin (BSA) was used as a standard protein (Sigma-Aldrich, USA) for this assay.

6.3.6 Effect of molasses on liver enzymes

The liver enzymes, ALP, AST and ALT were screened to determine the potential hepatotoxicity of sugar cane molasses. Commercially available diagnostic kits (BioQuant, CA) were used to measure the activity of the above mentioned liver

enzymes. Kits contained all components necessary for the assay and all procedures were performed using 96-well microtitre plates (Nunc, Serving Life Science, Denmark)

For the ALP assay, 10 µl of plasma samples was dispensed into wells of a microtitre plate. The working reagent (100 µl), prepared according to the manufacturer's guidelines was then added into all wells. Absorbance readings were taken immediately at 405 nm using a plate reader.

To determine AST levels, 20 µl of blood plasma samples was added to the wells of a microtitre plate. The assay was standardised using the AST calibrator (lyophilized serum with AST) supplied with the kit. A two fold serial dilution of the standard (20 µl/well) in distilled water was performed on the plate. Warm AST substrate (50 µl/well) was then added to all wells and the plate incubated for 10 minutes at 37 ºC. Following the incubation period, 50 µl of AST color reagent was dispensed into all wells. The reaction was stopped after 10 minutes (0.1 N HCL) and the absorbance read at 510 nm.

For the ALT assay, samples were added to the wells of a microtitre plate (20 µl/well). This assay was standardised using the ALT calibrator (solution of sodium pyruvate in 100 mM phosphate buffer at pH 7.4) supplied with the kit. A doubling dilution of the standard (20 µl/well) in distilled water was performed on the plate. ALT substrate (50 µl) was then dispensed into all wells and the plate incubated for 30 minutes at 37 ºC. Subsequently, ALT color reagent (50 µl) was added to wells and incubated for a further 10 minutes, after which the reaction was stopped with 200 µl/well of color developer (0.5 N sodium hydroxide). The plate was read immediately at an absorbance of 510 nm.

6.3.7 The effect of molasses on the male reproductive system

The experimental procedure used for this part of the study was similar to that previously described (Rahiman and Pool, 2010b).

At the end of the two month exposure period, treated and untreated animals (Treatment group 1 and Control group 1) were sacrificed by cervical dislocation and their testes were dissected out under aseptic conditions. The testis of each mouse was finely minced and suspended in 2 ml of culture medium, which comprised of RPMI-1640 (Sigma-Aldrich, USA) supplemented with 0.2 % bovine serum albumin (Sigma-Aldrich, USA), 1 % glutamax and 1 % mixture of streptomycin, penicillin and fungizone (Sigma-Aldrich, USA). Cell debris was removed and the supernatant containing cells was centrifuged at 1000 x g (Super mini centrifuge, MiniStar Plus, Hangzhou Allsheng Instruments, China) for 10 minutes. The cell pellet was resuspended in a final volume of 5 ml and incubated for 1 hour at 37 ˚C with 5 % CO_2. At the end of the incubation period, the cell suspension was centrifuged at 1000 x g for 10 minutes and the cell pellet was resuspended in 5 ml of fresh medium. Cells were incubated for a further 30 minutes at 37 ˚C with 5 % CO_2, after which cells were centrifuged as previously described and resuspended in 5 ml medium to give a final concentration of 1 x 10^6 cells/ml.

Cells (100 μl) were then dispensed into wells of a 96-well tissue culture plate (Nunc, Serving Life Science, Denmark). Cell preparations were either stimulated or not stimulated with 10 mU/ml human luteinizing hormone (100 μl per well) (LH) (Sigma-Aldrich, USA) and incubated at 37 ˚C with 5 % CO_2 for 4 hours. The control used throughout this study comprised of cells in medium only. At the end of the incubation time, culture supernatants were harvested and screened for testosterone synthesis

146

using commercially available testosterone enzyme linked immunosorbent assay (ELISA) kits (DRG Instruments GmbH, Germany). These kits supplied all the necessary reagents for the assay and the procedure was conducted according to the manufacturer's instructions (refer to 4.3.5).

6.3.8 Statistical analysis

All data was statistically determined via one-way ANOVA using SigmaStat software (Systat Software Inc., USA). Results obtained for parameters such as body weight, molasses and water intake were expressed in terms of the mean ± standard error of the mean (SEM) for six mice per group (n=6). All assays were performed in triplicate to prevent statistical errors and data was expressed as the mean ± SEM of three replicates for six mice per group (n=18).
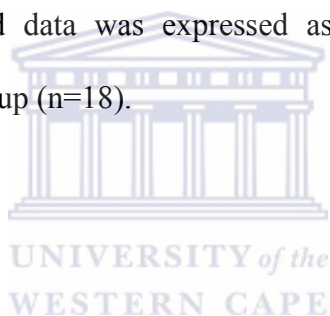
6.4 RESULTS

Effect of daily oral consumption of sugar cane molasses on various parameters in mice

6.4.1 General observations

The behaviour of molasses-treated animals appeared to be more aggressive when compared to the control group. For this reason, treated animals were further separated and housed as two mice per cage. It was also observed after day 40 of treatment with molasses that mice exhibited signs of loose faeces.

6.4.2 Body weight (BW), water and molasses intake

The effect of molasses on body weight (g) is presented in Table 6.1. All data are expressed as the mean and standard error of the mean (SEM). Both control and molasses treated groups appeared to have gained weight from the start of the experiment to the end of the 60-day period. However, the weight gain from day 0 up to day 60 was not statistically different for the control or treated groups ($P>0.05$). The average drinking volumes obtained for molasses and control mice are indicated in Table 6.2. Results show for the duration of day 20 up to day 60, treatment groups consumed significantly more fluid in comparison to the control groups ($P<0.05$).

Table 6.1 Body weight (g/mouse) of molasses treated and control mice for exposure period

Group	D0	D10	D20	D30	D40	D50	D60
Control	29.02 ± 0.50	29.24 ± 0.39	30.13 ± 0.39	30.38 ± 0.51	30.87 ± 0.57	30.95 ± 0.51	31.68 ± 0.48
Molasses	27.54 ± 0.70	28.40 ± 0.58	29.13 ± 0.61	29.25 ± 0.58	29.39 ± 0.57	29.42 ± 0.77	30.01 ± 0.62

All values are expressed as the mean and standard error of the mean (SEM), where n=6 for the control and treatment groups. Body weight was recorded for the exposure period D0-D60 i.e. Day 0 (initial BW at the start of the experiment) up to Day 60 (final BW at the end of the experiment).

Table 6.2 Drinking volumes (ml/mouse) obtained for molasses treated and control mice for exposure period

Group	D10	D20	D30	D40	D50	D60
Control	5.17 ± 0.06	4.94 ± 0.05	5.16 ± 0.16	5.47 ± 0.08	4.94 ± 0.03	5.44 ± 0.08
Molasses	6.18 ± 0.45	7.79 ± 0.80*	8.42 ± 0.78*	8.57 ± 0.61*	11.26 ± 0.85*	12.83 ± 0.82*

All values are expressed as the mean and standard error of the mean (SEM), where n=6 for the control and treatment groups. Oral intake of each group was monitored for the exposure period D10-D60 i.e. Day 10 (oral consumption at day 10) up to Day 60 (oral consumption at end of experiment). An asterisk (*) designates the statistical difference when compared to the control ($P < 0.05$)

6.4.3 Effect of molasses on serum biomarkers

Table 6.3 presents the average data obtained for the effects of molasses on various serum biomarkers at the end of the exposure period. Immunised and non-immunised animals that were exposed to molasses treatment showed no significant difference to their respective control groups when measuring hematocrit levels and total protein (P>0.05). Exposure of molasses to mice also revealed no cytotoxic effects when compared to control mice (P>0.05). Results determining the effects of molasses on liver enzyme activity at the end of the exposure period are indicated in Table 6.4. AST, ALT and ALP are well-established biomarkers of liver damage (Field et al., 2008). Immunised and non-immunised, molasses treated mice showed no difference in plasma AST, ALT and ALP levels when compared to untreated mice (P>0.05).

Table 6.3 Effect of daily oral administration of molasses on serum biomarkers in mice

Parameters	Immunised animals		Non-immunised animals	
	Treated	Control	Treated	Control
Hct (%)	52 ± 1.02	52 ± 2.08	50.78 ± 0.49	51.44 ± 0.29
LDH (%Toxicity)	2.82 ± 0.19	2.96 ± 0.29 (-ve control)	3.05 ± 0.229	3.03 ± 0.11 (-ve control)
TP (μg/ul)	27.90 ± 0.93	29.91 ± 0.98	22.00 ± 0.38	22.86 ± 0.67

All values are expressed as the mean and standard error of the mean (SEM) of three replicates (n=18). Hct, hematocrit; LDH, lactate dehydrogenase activity; TP, total protein. An asterisk (*) designates the statistical difference when compared to the control (P<0.05).

Table 6.4 Effect of daily oral administration of molasses on liver enzyme activity

Enzyme levels IU/L	Immunised animals		Non-immunised animals	
	Treated	Control	Treated	Control
AST	13.12 ± 0.62	11.58 ± 0.63	10.42 ± 0.67	9.79 ± 0.679
ALT	13.78 ± 1.10	15.21 ± 1.30	12.96 ± 0.84	12.70 ± 0.87
ALP	17.20 ± 1.03	16.48 ± 0.81	14.33 ± 15.08	15.08 ± 1.07

All values are expressed as the mean and standard error of the mean (SEM) of three replicates (n=18). AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. An asterisk (*) designates the statistical difference when compared to the control (P<0.05).

6.4.4 The effect of molasses on the immune system

Figure 6.1 illustrates a typical standard curve used to calculate the antibody titre for molasses-treated and untreated mice. A power trendline with a correlation coefficient of 0.975 was obtained for the relationship between dilution factor versus OD at 450 nm. The measurement of IgG anti-antigen was used to determine the impact of molasses on humoral immunity. Results presented in figure 6.2 show that serum levels of IgG anti-antigen for the treatment group was significantly reduced when compared to the control group ($P<0.05$).
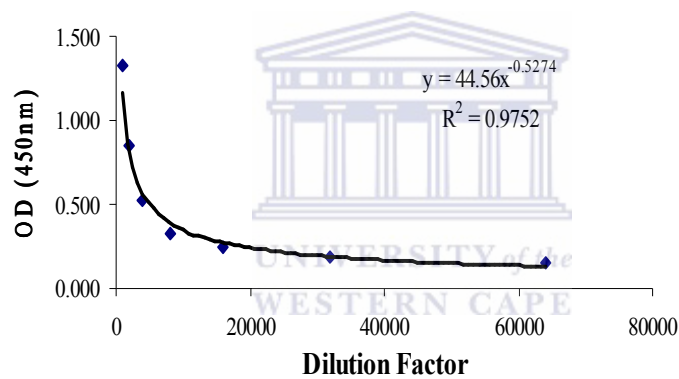
Figure 6.1 Standard curve used to determine IgG anti-antigen titre in molasses treated and untreated mice. A power trendline with a correlation coefficient of 0.975 was obtained.
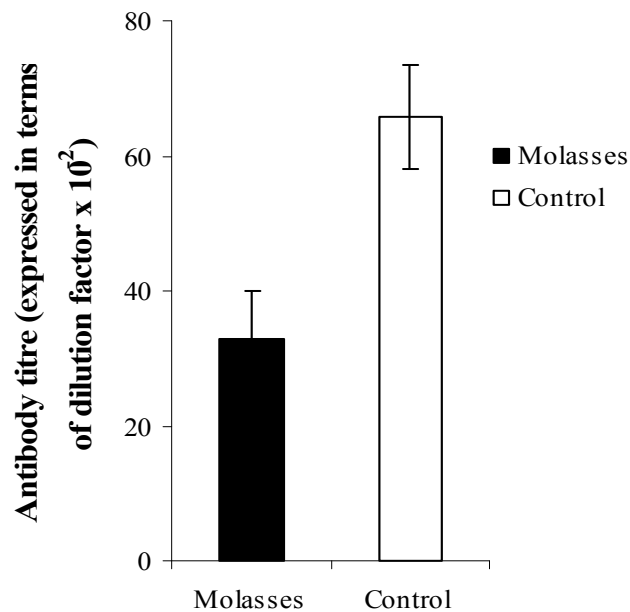
Treated and untreated groups

Figure 6.2 The effect of sugar cane molasses on IgG anti-antigen titre in molasses treated and untreated mice. Each point is the mean ± SEM (standard error of the mean) of three replicates (n=18). An asterisk (*****) indicates statistical difference when compared to the control (P<0.05).

6.4.5 The effect of molasses on the male reproductive system

Animals that were either treated or untreated with molasses *in vivo,* were screened for testosterone synthesis using a previously established *in vitro* assay (Rahiman and Pool, 2010b). The concentration of testosterone produced by cells incubated with samples were calculated using a standard curve similar to that presented in figure 4.1 Figure 6.3 illustrates the *ex vivo* effect of molasses on testosterone secretion using testicular cell cultures. Results show that exposure of mice to molasses significantly increase LH induced testosterone production when compared to the control animals (P<0.05). No difference was seen in the testosterone production of molasses treated and untreated mice in the absence of LH stimulation (P>0.05).
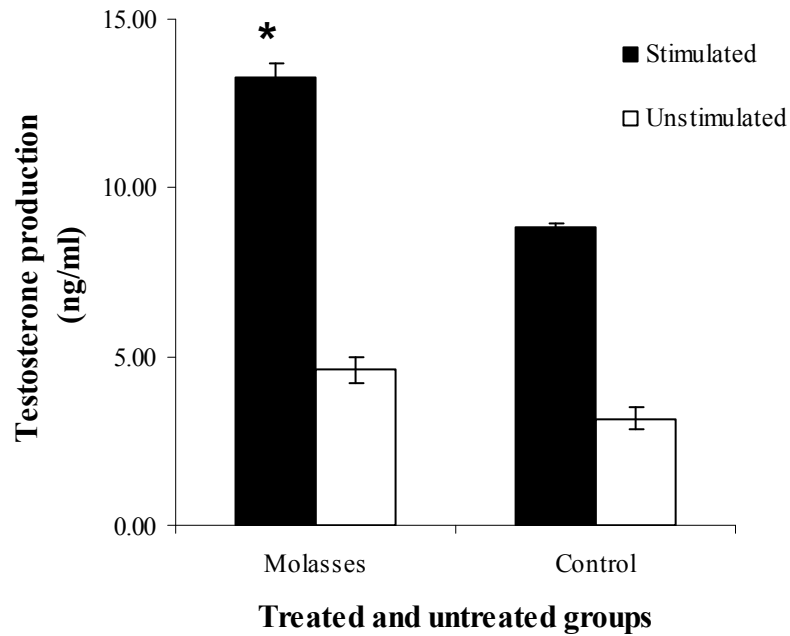
Figure 6.3 The effect of sugar cane molasses on testosterone synthesis in testicular cell cultures. Each point is the mean ± SEM (standard error of the mean) of three replicates (n=18). An asterisk (*****) indicates statistical difference when compared to the control (P<0.05). Stimulated=■; Unstimulated=☐.
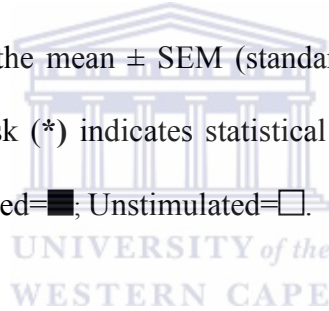
6.5 Discussion

This study shows that molasses does not have any significant effect on body weight of animals. However, fluid intake was significantly increased in the molasses group and this appeared to provoke the onset of loose stools observed in treated animals. This observation is consistent with previous research. Results also showed that total protein levels of the experimental and control groups were similar. This supports the suggestion that the effects seen in the experimental group were due to molasses consumption and not as a consequence of dehydration. Studies have shown that cattle fed diets containing moderate to high levels of molasses suffer with loose faeces, which is often associated with diarrohea. Sucrose and potassium in molasses have been implicated as causal factors in the development of digestive problems seen in animals and may also contribute to the laxative property of this compound (Pate, 1983).

The haematopoietic system is a susceptible target of various toxic compounds and has been used as an indicator of potential adverse effects on the physiology and pathology of the body (Li et al., 2010). This study shows that molasses had no significant effect on haematocrit levels, suggesting that molasses does not affect circulating red blood cells but rather maintains the normal physiological range of these cells. Supportive of previous data, results show that exposure of molasses *in vivo* does not initiate any cytotoxic effects that may affect organ system functionality (Rahiman and Pool, 2010a). The molasses-treated group also demonstrated no significant effect on serum TP levels when compared to the control group, implying that it has no effect on protein synthesis.

Analysis of liver enzyme activity revealed no significant difference between treated and untreated groups for the biomarkers ALP, AST and ALT. Hepatocellular injury is usually characterised by an elevation in cytosolic levels of these liver enzymes (Field et al., 2008). Molasses does not alter levels of these marker enzymes, indicating that it has no adverse effect on hepatocyte function or metabolism.

An effective immune response is able to detect invading pathogens and defend against infection. The immune response of humans appears to be similar to that produced by mice. Immune cells are constantly synthesised and travel throughout the body of humans and mice. Leukocytes are cells involved in inducing a specific immune response and their function can be monitored experimentally by determining their response to antigens or their potential to synthesise specific cytokines and antibodies (Winans, 2010). This study investigated the effects of molasses on the humoral immune system by assessing antibody production in immunised animals. Results show that molasses significantly inhibited the IgG response to antigen in treated animals. This result was in contrast to our *in vitro* data, which indicated that molasses stimulates humoral immunity (Rahiman and Pool, 2010a). Our current findings suggest that *in vivo* exposure to molasses reduces levels of IgG against the antigen, which may be associated with an inability to produce an effective humoral immune response when challenged with an antigen. It has been reported that IgG plays a defensive role against bacterial pathogens such as serogroup B and *Neisseria meninigitis* in humans (Lin et al., 2006). Hence, a reduced humoral response induced by molasses intake may be associated with an increased susceptibility to the above-mentioned extracellular pathogens. The immunosuppressive potential of molasses as a result of prolonged exposure, may also enhance the risk of already

immunocompromised individuals (patients suffering from cancer or AIDS) to the development of infection.

Testosterone production is an important index of potential reproductive toxicity. Any changes resulting in suppression of testosterone synthesis may lead to detrimental effects on reproductive processes such as spermatogenesis. Therefore, this steroid hormone is vital in maintaining the function and organization of accessory sex glands in males (Karacaoğlu and Selmanoğlu, 2010). Data from our *in vitro* studies demonstrated the stimulatory effect of molasses on testosterone biosynthesis in male mice (Rahiman and Pool, 2010b). Consistent with this result, this study shows that under stimulatory conditions molasses induces a significant elevation on plasma testosterone *in vivo*. For many years, traditional healers have used plants, fungi and insects to enhance libido and improve fertility. The fungal parasite, *Cordyceps sinensis* has been used extensively by the Chinese as a tonic herb to improve and restore sexual performance. Studies have shown that *Cordyceps sinensis* exhibits an enhancing effect on testosterone secretion both *in vitro* and *in vivo* (Hsu et al., 2003). Hsu et al. (2003) suggests that the stimulatory effect of *Cordyceps sinensis* on testosterone synthesis may prove to be beneficial in treating males that suffer from reproductive dysfunction as a result of inadequate testosterone synthesis (Hsu et al., 2003). Since molasses displays a similar enhancing effect on testosterone secretion, the use of molasses may also be potentially favorable in improving reproductive function and sexual performance.

6.6 Conclusion

It can be concluded from this study that molasses may have potential adverse or beneficial effects on certain physiological systems. The immunostimulatory effect of molasses exhibited *in vitro,* was not observed *in vivo*. Findings for this study show that exposure to molasses *in vivo* produces an immunosuppressive effect. This result suggests that molasses may reduce the humoral immune response when administered daily over a prolonged period. Results determining the effect of molasses on the male reproductive system demonstrate that it has the ability to enhance testosterone production *in vivo*. This data validates the outcome obtained in our *in vitro* study and further promotes the use of molasses as a supplement to enhance testosterone production, thereby improving reproductive health. Data achieved in this study provides valuable information on the biological activity of molasses on various physiological systems that may prove to be useful in further *in vivo* testing.
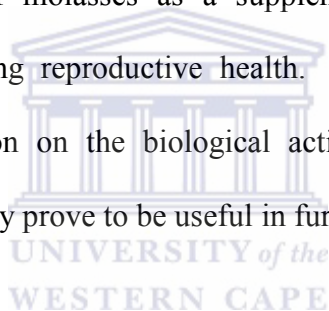
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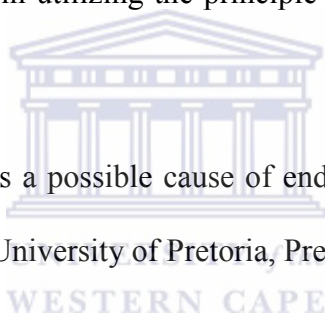
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161

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Chapter 7

General conclusion

Natural and artificial sweeteners elicit the enjoyable sensation of sweetness. The preference for sweet taste by the human population has created a huge demand for the manufacture of such products by the food industry. Sweeteners have become an integral dietary ingredient and its uses have expanded worldwide. Historically, sugar has been the predominant sweetener used to increase palatability of various food items and medications. In addition, natural sweeteners such as sugar cane molasses which is generally regarded as 'safe' or 'harmless' has been used for its proposed health benefits. Development in the food sector has lead to the wide-scale production of various artificial sweeteners that provides the consumer with sweet taste offered by sugar but without the added calories that support adverse health effects. Reports on the use of both natural and artificial sweeteners have suggested that their inclusion in the human diet may have adverse consequences on human health. However, contradicting evidence has demonstrated the safe use of both these products.

The increased use of sweeteners has raised much concern amongst the public and regulatory authorities. New or improved food products are continually emerging into the food sector and need to be subjected to toxicity testing to evaluate their safety. Since artificial and natural sweeteners have become entrenched in the food industry and used extensively, it is necessary that we determine its effects on the human body. This study focused on the impact of commercially available and frequently used natural and artificial sweeteners on several physiological systems.

The *in vitro* procedures used in this study proved to be a sensitive, cost-effective and efficient method of screening for potential adverse effects of sweeteners on organ systems. Results obtained from these studies show that certain artificial sweeteners have an effect on important biomarkers regulating the immune response and ovarian steroidogenesis. These findings suggest that such sweeteners may be implicated in the development of certain health conditions. However, further studies are warranted to substantiate these reported effects.

The natural sweetener, sugar cane molasses was the only sample to have a significant impact on all tested, organ systems *in vitro*. To validate this outcome, we further investigated the effect of molasses on physiological systems by using *in vivo* and *in vitro* tests. Results showed that molasses induced a modulatory effect on biomarkers regulating the immune system and male reproductive processes. The immunosuppressive potential demonstrated by molasses *in vivo,* was in contrast to that observed in our *in vitro* tests. Nevertheless, molasses still shows the ability to alter the immune response, which may affect immune function. The *ex vivo* method proved to be very effective as it combined the advantages of using a whole animal with the benefits of *in vitro* testing to assess the impact of molasses exposure on the male reproductive system. The potential of molasses to increase testosterone levels was consistent with data obtained in our preliminary, *in vitro* study. This finding suggests that molasses may be beneficial in improving sexual performance and reproductive health.

Research investigating the impact of molasses on human health is limited. Results of this study demonstrate certain characteristics of molasses that may be linked to either adverse or beneficial effects on human health. Hence, this study provides valuable

information on the biological activity of molasses. However, a lot about the nature of this compound still remains unknown and further testing of molasses is required. Data achieved in this study may be useful in conducting future *in vivo* studies.

APPENDIX 1

Table 3.1 Percentage toxicity of molasses samples incubated with whole blood cultures

Molasses (ug/ml)	Stimulated		Unstimulated	
	Mean	Standard deviation	Mean	Standard deviation
0	2.3	0.04	2.4	0.02
12.5	2.3	0.07	2.2	0.07
25	2.3	0.03	2.4	0.04
50	2.2	0.01	2.5	0.06
100	2.4	0.07	2.4	0.01
200	2.4	0.01	2.4	0.00
400	2.4	0.02	2.3	0.05
800	2.3	0.03	2.2	0.06

A typical example of LDH (lactate dehydrogenase) values obtained for different subjects. All values are expressed as the mean and standard deviation for each treatment. An asterisk (*****) designates the statistical difference of a molasses sample when compared to the control ($P < 0.05$).

Table 3.2 Percentage toxicity of natural and artificial sweeteners incubated with whole blood cultures

Samples (ug/ml)	Stimulated		Unstimulated	
	Mean	Standard deviation	Mean	Standard deviation
Canderel™	2.7	0.10	2.8	0.11
Equal™	2.7	0.10	2.7	0.10
Natreen™	2.8	0.17	2.7	0.07
Sweetex™	2.7	0.01	2.9	0.21
Splenda™	2.6	0.23	2.7	0.28
Swheet™	2.5	0.21	2.8	0.22
Brown sugar	2.7	0.22	2.9	0.07
White sugar	2.7	0.21	2.8	0.06
Molasses	2.6	0.03	2.8	0.17
Control	2.7	0.22	2.8	0.23

A typical example of LDH (lactate dehydrogenase) values obtained for different subjects. All values are expressed as the mean and standard deviation for each treatment. An asterisk (*****) designates the statistical difference of a sweetener sample when compared to the control ($P < 0.05$).

Table 4.1 Percentage toxicity of molasses samples incubated with testicular cell cultures

Molasses (ug/ml)	Stimulated		Unstimulated	
	Mean	Standard deviation	Mean	Standard deviation
0	1.2	0.03	1.2	0.01
12.5	1.1	0.04	1.1	0.03
25	1.0	0.21	0.9	0.08
50	1.3	0.10	1.1	0.02
100	0.9	0.20	1.0	0.06
200	1.1	0.08	0.9	0.12
400	1.2	0.01	1.1	0.09
800	1.1	0.1	1.0	0.24

A typical example of LDH (lactate dehydrogenase) values obtained for testicular cell cultures. All values are expressed as the mean and standard deviation for each treatment. An asterisk (*****) designates the statistical difference of a molasses sample when compared to the control ($P<0.05$).

Table 4.2 Effect of molasses samples on estradiol synthesis in stimulated and unstimulated testicular cell cultures.

Molasses (ug/ml)	Percentage estradiol (E_2) (pg/mg protein)			
	Stimulated		Unstimulated	
	Mean	Standard deviation	Mean	Standard deviation
0	-1.8	0.26	-1.2	0.28
12.5	-1.8	0.47	-1.2	0.34
25	-1.9	0.45	-1.3	0.52
50	-2.2	0.43	-1.1	0.65
100	-2.1	0.40	-1.9	0.49
200	-2.2	0.25	-1.2	0.55
400	-1.9	0.29	-1.3	0.61
800	-2.3	0.43	-1.2	0.72

For percentage E_2, values are indicated as the mean and standard deviation for each treatment. An asterisk (*****) represents the statistical difference of a sweetener sample in comparison to the control ($P<0.05$).

Table 4.3 Effect of natural and artificial sweeteners on estradiol synthesis in stimulated and unstimulated testicular cell cultures.

Samples (ug/ml)	Stimulated		Unstimulated	
	Mean	Standard deviation	Mean	Standard deviation
Canderel™	-2.1	0.69	-1.6	0.71
Equal™	-2.6	0.46	--1.6	0.76
Natreen™	-2.2	0.45	-1.9	0.61
Sweetex™	-2.3	0.49	-1.7	0.76
Splenda™	-2.4	0.63	-1.7	0.68
Swheet™	-2.6	0.55	-1.6	0.77
Brown sugar	-2.4	0.39	-1.7	0.8
White sugar	-2.3	0.43	-1.5	0.65
Molasses	-2.3	0.52	-1.7	0.72
Control	-2.3	0.59	-1.6	0.75

For percentage E_2, values are indicated as the mean and standard deviation for each treatment. An asterisk (*****) represents the statistical difference of a sweetener sample in comparison to the control ($P<0.05$).

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