AMERICAN UNIVERSITY OF BEIRUT

THE EFFECT OF ASPARTAME AND SUCRALOSE CONSUMPTION ON FOOD INTAKE AND BODY COMPOSITION OF RATS

by
FIDELE HAMAD EL MASRI

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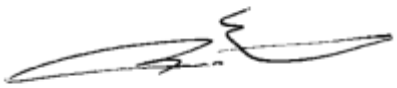

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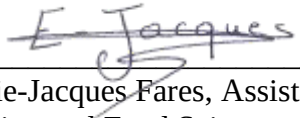

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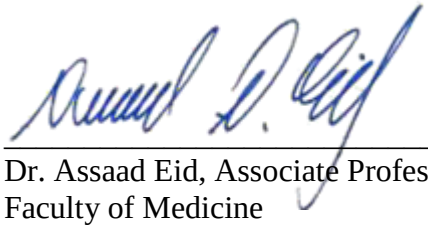

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AN ABSTRACT OF THE THESIS OF

Fidele Hamad El Masri for Master of Science
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Nowadays, the world is witnessing a high prevalence and a dramatic increase of overweight and obesity. It became a major concern in global health because it is even threatening children under five years of age, it is associated with metabolic abnormalities, and it is considered a major risk factor for non-communicable diseases. The free and added sugar are considered one of the major contributors to the positive energy balance that contributes to increased adiposity and obesity. Therefore, artificial sweeteners (AS) were introduced in the food and beverages industries as a substitute for sugar. AS were expected to be healthy substitute for sugar to prevent and treat the burden of obesity and its metabolic related diseases. However, many controversies exist in the literature regarding the metabolic effects of AS. The objective of this rodent study is to assess the metabolic effects of consumption of usual amounts of aspartame and sucralose (commonly used AS) in food and/or in water. The study mimics real-life situations since sucralose and aspartame are present with moderate amounts in a variety of food and beverages. 48 adult male rats were divided into 7 groups (1 control group+ 6 interventional groups). The Control group was fed for 8 weeks a regular starch diet accompanied with regular water. Whereas the interventional groups were fed for 8 weeks either (1) a regular starch diet, with aspartame-/ sucralose- sweetened water (2) or aspartame-/sucralose- sweetened starch diet with regular water, (3) or aspartame-/sucralose- sweetened starch diet, with aspartame-/ sucralose- sweetened water. After that, rats were sacrificed, and metabolic analysis was performed. Comparing the consumption of starch without AS vs. starch sweetened with different doses of aspartame and sucralose, the AS aspartame and sucralose were shown to be significantly associated with an increase in body weight and fat mass, accompanied with a decrease in lean mass independently from food intake. The severity of the effect depends on the dose of the AS. Smaller doses of sucralose did not have a significant effect on body weight, but they did not fail to alter the body composition and increase the fat mass. Although the effect on serum glucose, insulin, and lipid profile and kidneys was not significant, however, it is believed that a longer period of consumption could show significant results. Sucralose and aspartame were tested for safety but limited work has been done on their efficacy.

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ILLUSTRATIONS

TABLES

ABBREVIATION

°C: Degree Celsius

>: Greater than

<: Less than

-: Minus

/: Per

%: Percent

x: Times

ADI: Acceptable Daily Intake

AS: Artificial Sweeteners

Asp: aspartame

BUN: Blood Urea Nitrogen

CCK: Cholecystokinin

EDTA: Ethylenediaminetetraacetic acid

dl: Deciliter

ELISA: Enzyme-Linked Immunosorbent Assay

et al.: and others

FDA: Food and Drug Administration

g: Gram

GIP: glucose-dependent Insulinotropic Peptide

GI tract: Gastrointestinal Tract

GLP-1: Glucagon-like Peptide 1

G-PCR: G Protein-coupled Receptor

GRAS: Generally Recognized As Safe

HDL: High-Density Lipoprotein

HOMA: Homeostasis Model Assessment

IAP: Intestinal Alkaline Phosphatase

IACUC Institutional Animal Care and Use Committee

LDL: Low-Density Lipoprotein

LPS: Lipopolysaccharide

mg: Milligrams

ml: Milliliter

ng: Nanograms

NODs: Nucleotide Oligomerization Domains

PYY: Peptide YY

Scl: sucralose

SD: Standard Deviation

T1R: Taste 1 Receptor

T2R: Taste 2 Receptor

TC: Total Cholesterol

TG: Triglyceride

TLRs: Toll-like Receptors

SPSS: Statistical Package for The Social Sciences

VLDL: Very Low-density Lipoprotein

WHA: World Health Assembly

WHO: World Health Organization

To Wafaa and Hamad, my beloved parents

CHAPTER I

INTRODUCTION

Nowadays, the world is witnessing a high prevalence and a dramatic increase of overweight and obesity. In the last five decades, obesity has nearly tripled worldwide. This epidemic remains the main cause of premature death. It is affecting all age groups even children under five years of age(1, 2). In 2016, 39% of adults(≥18 years of age) were overweight, and 13% were obese.(2). The World Health Organization (WHO) defines overweight and obesity as excessive or abnormal fat accumulation that can present a risk to health. Overweight is a slightly increase in body weight compared with normal weight, which is resulted from an increased body fat percentage. Whereas, obesity is a chronic disease condition with high morbidity and mortality risks, which is a pronounced form of overweight (1). The body mass index (BMI) is used to categorize overweight and obesity in adults. It is defined as a person's weight in kilograms divided by the square of his height in meters (kg/m2). A person with a BMI greater than or equal to 25 is considered overweight; and a person with a BMI greater than or equal to 30 is obese. The fundamental contributor of obesity is an energy imbalance between energy intake and energy expenditure. This imbalance is due to an interaction between individual factors (for example: genetics, epigenetics, or the gut–brain–hormone axis), along with the environmental and social factors (such as changes in both dietary habits and physical activity patterns as a result of an obesogenic environment due to the nutrition transition). The environmental and social factors can increase the genetic susceptibility to obesity(1). Therefore, many studies support that obese individuals should not be blamed and stigmatized for their obesity, because the

whole environment that they live in is obesogenic (transport systems, global food system, food environment, density of fast food chains, food industries, and media)(1, 3). In addition, obesity constitutes a major health and economic challenge. In fact, a study conducted by Yusefzadeh et al. has shown that obesity costs accounts for 31.8% of direct costs (costs for the health systems), and 68.1% of indirect costs (costs for productivity loss due to illness-related absenteeism, premature retirement or premature death)(4). Obesity is associated with an increased risk of diabetes, cardiovascular diseases, cancer, dementia, bone diseases, liver diseases, mental diseases, and sexual diseases(1, 5). As a results, many countries started to look for answers about how to reverse the rising tide of adult and childhood obesity. In spite of all the efforts, prevention and treatment strategies -both at the individual and population levels- have failed in the long term. Studies have shown that the major contributor to this dramatic increase in energy intake (positive energy balance), resulting in increased adiposity and obesity is mainly the consumption free and added sugar (especially in sweetened beverages)(1, 6, 7). In 2004, the 57th World Health Assembly (WHA) endorsed the WHO Global Strategy on Diet, Physical Activity and Health. It recommended the individuals to limit consumption of free and added sugar throughout their lifecourse (strong recommendation), and advised the governments to take corrective actions and develop policies targeting the food and beverages industries that use sugar, in order to promote health. The latest WHO guidelines recommend all age groups to reduce their daily intake of free sugars to less than 10% of their total energy intake (strong recommendation). A further reduction to below 5% or roughly 25 grams (6 teaspoons) per day would provide additional health benefits (conditional recommendation)(8). Sugar has a negative effect on health because dietary sugar upregulates hepatic uptake

and metabolism of fructose, resulting in fatty liver, dyslipidemia, reduced insulin sensitivity, and high uric acid(9). Therefore, artificial sweeteners (AS) were introduced in the market and the food and beverages industries as a substitute for sugar. Their usage has increased a lot in the past two decades. Nowadays, studies have shown that the consumption of AS is 25% in children and 41% in adults. The AS are considered food additives. They mimic the sweet taste of natural sugars (glucose, fructose, galactose, and sucrose), with a negligible amount of energy. The taste is important for palatability and acceptability of food. It gives a sensation of enjoyment and pleasure. The Food and Drug Administration (FDA) has approved the use of 6 artificial sweeteners (acesulfame-potassium, aspartame, advantame, neotame, saccharin, and sucralose) and marked them as Generally Recognized As Safe (GRAS). In our study, we will focus only on sucralose (non-nutritive sweetener) and aspartame (nutritive sweetener), due to their high demand in the industries. Aspartame and sucralose are considered high-intensity sweeteners, their sweeting power (measured relatively to sucrose) is 160-220x and 600x respectively(10). Aspartame and sucralose contribute in negligible amount of caloric intake; therefore, science and industries were expecting that they will help in reducing the burdens of metabolic syndrome, obesity, and insulin resistance. However, many controversies exist about the safety and the health benefits of sucralose and aspartame since the incidence of obesity and type 2 diabetes are increasing parallelly to their increased consumption over the past two decades.

CHAPTER II

LITTERATURE REVIEW

A. Aspartame

Aspartame (L-aspartyl-L- phenylalanine methyl ester) is a methyl ester of aspartic acid and phenylalanine dipeptide. It was discovered by accident in 1965 and approved by the FDA in 1981 for use in specific foods, and few years later in 1983 for use in soft drinks. The acceptable daily intake (ADI) of aspartame is 50 mg/kg body weight. Aspartame's chemical structure exists in two forms (α and β); however, only the α form of aspartame provides the sweet taste. Although aspartame provides 4 kcal/g when metabolized, but the quantity of aspartame used to give a sweet taste is so small that its caloric contribution is negligible. This odorless white crystal is hydrophilic and heat sensitive (cannot be used in cooking and baking). It is most stable at a pH between 4-5, with a half-life of over 250 days at 25◦C. Aspartame is sold under the brand names NutraSweet®, Equal®, Canderel®, and Sugar Twin®. Research has been examining the intestinal absorption and metabolism of aspartame. In the upper gastrointestinal tract, under the effect of esterases and peptidases, aspartame is metabolized into 3 major compounds: aspartic acid, phenylalanine, and methanol; and other break down products including formaldehyde, formic acid, and diketopiperazine. After that, those compounds will be metabolized like they would be derived from other food sources. They will be absorbed by intestinal mucosal cells where they will be hydrolyzed to their components, transported across the wall of the small bowel, go into the circulation, and finally reach the liver. Research studies have been intensively examining the safety of aspartame.

The FDA claims that Aspartame is one of the most profoundly studied substances in the food industry.

B. Sucralose

On the other side, Sucralose (4,1',6'-trichlorogalactosucrose) is also a high intensity non-nutritive sweetener, with an ADI of 5mg/kg body weight. It is a disaccharide in which three chlorine molecules replace three hydroxyl groups on the sucrose molecule. It was approved by FDA in 1998. Sucralose is sold under the brand names Splenda®. Unlike aspartame, sucralose is heat stable; thus, it is used in cooking and baking. Also, sucralose was found in sewage treated water unchanged, since it is resistant to Ph. and water treatments(11). Nevertheless, some studies have found that sucralose decomposition starts at a temperature of 119 ℃, resulting in toxic compounds(12). Furthermore, research suggests that most sucralose (85%) is not absorbed and is excreted unchanged in feces. the sucralose that is absorbed is excreted unchanged in urine(13). However, other studies argue that traces of sucralose metabolites were found in feces, which indicated that sucralose is metabolized and absorbed. Also, sucralose bioaccumulates in organs such as kidneys and adipose tissues. Data on sucralose metabolism remains inconsistent and controversial.

C. Mechanism of Sweet Taste Perception

Liking of sweet taste is innate, it refers to food reward. Nevertheless, perception of sweetness and preferred level of sweetness differs from one individual to another. It depends on the sweet taste receptors present on the tongue (taste buds). Some studies suggest that it is related to an interaction between environmental exposures and

genetics(10, 14). Sweets consumption is addictive, it has downstream effects on behavioral and neurochemical pathways leading to the “reward phenomenon”. In fact, sweets can stimulate feeding, even when energy requirements are met, leading to excess energy intake, and obesity(14).

Sweet taste perception first starts in the oral epithelium, mostly at the level of the tongue, where the type 2 taste receptors (TCRs) interact with the sweet food and beverages consumed. The TCRs are G-protein coupled receptors (G-PCRs), they are divided into 2 families: the taste 1 receptor (T1R), and the taste 2 receptor (T2R). However, only the T1R receptors interact with sweetness. The T1R are divided into two subunits: T1R2, and T1R3. T1R2 and T1R3 respond to sugar (sucrose, fructose, galactose, glucose, and maltose), to artificial sweeteners, and to other compounds (some amino acids, and sweet proteins). Binding one subunit stimulates the sweet response, but binding a second subunit increases the response. After that, a transduction mechanism translates the sweet taste message via the nervous system to the brain (hypothalamus and amygdala) that perceives the sweet taste, resulting in a feeling of satisfaction. Research studies suggest that this mechanism results in increased intracellular calcium (Ca) and neurotransmitters release. In fact, since the sweet taste receptors are G-PCRs, they induce downstream activation of the second messenger system (an intracellular signaling molecule transit from a receptor to a target). Once a sweet compound binds to the sweet receptors T1R2 and T1R3, a signaling protein molecule α-gustducin gets activated, and it stimulates transducing. Both of α-gustducin and transducing increase phosphodiesterase (PDE), decrease intracellular cyclic adenosine monophosphate (cAMP) levels, increases phospholipase Cβ2 (PLCβ2), thereby inositol 1,4,5-trisphosphate and diacylglycerol gets activated. Therefore, these

compounds activate the transient receptor potential cation channel subfamily M member 5 (TRPM5), which increases intracellular calcium and neurotransmitter release. Research studies found that knocking out α-gustducin in rodents reduces, but not eliminates taste responses to sweet taste.

Additionally, sweet taste receptors have also been found in the gastrointestinal (GI) tract (endocrine cells L and K). As a proof research studies using sweet-taste inhibitors resulted in a decrease in glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) secreted by the endocrine L cells, but cholecystokinin (CCK) secreted by the I cells was not affected. Thus, sweet taste receptors exist in the intestine and they are associated with the regulation of the secretion of incretin hormones GLP-1 and PYY. Sweet taste receptors were additionally found in various other organs including the biliary and respiratory tracts, adipose tissues, kidney, bladder, brain, heart, and the pancreatic ß- islet cells. Therefore, AS might have additional questionable effect in the body(9).

D. AS Consumption

Studies show that that the average of AS intake among adults is below the ADI(10). However, AS exist in food and beverages in hidden forms (mouthwash, toothpaste, sauces, frozen desserts, pudding, yogurt...), and it is challenging to assess the total AS consumption. AS have become part of the westernized diet that is spreading across the world. It is hard to assess and control the intake. Although aspartame is labeled on the food and beverages items; however, very few consumers read the label to evaluate their AS intake. Mostly individuals with phenylketonuria are interested to

monitor their aspartame intake, because it contains phenylalanine, and they don't have the enzymes to metabolize it; thus, aspartame can be fatal for them.

E. Metabolic Effects of AS

Many controversies exist in the literature about the metabolic effects of AS.

1. Energy intake

Excessive energy intake contributes to obesity. Hence, AS (energy-free aspartame and sucralose) are used excessively in food and beverages industries in order to substitute the energy dense food and beverages that contain sugar. In theory, this will help in reducing energy intake, maintaining a healthy body weight among individuals that enjoy the sweet taste, since the consumption of sweets is addictive(10, 14). Many studies have shown that replacing sugar-sweetened beverages, but not water with aspartame-sweetened drink contribute in a significant decrease in energy intake(15). Other studies on both adults and children have shown a neutral effect of sucralose on energy intake, but long-term studies are needed(10). On the other hand, multiple studies argued that AS failed to meet the expectations of reducing energy intake. For instance, Mitsutomi et al. indicated that aspartame did not help to lower energy intake, in an animal study(16). In fact, adding sucralose or aspartame to unsweetened food and beverages was shown to increase the intake in both animal and human studies(14, 17). The negative effect of AS (including aspartame and sucralose) on energy intake is more pronounced than the effect of sugar(14, 18). AS were shown to lead to overconsumption during subsequent meals; thus leading to excessive caloric intake(14). Some studies suggest that when people are aware they are consuming AS, they consciously

overcompensate(14). Research needs to focus more on special subjects, and have enough number of them in the studies. For example, diabetics usually have an intake of AS higher than the usual level. Also, pregnant women and children are considered a special population. Therefore, it is advised for research to shed a light on the safety of AS consumption among the special population, especially that AS exist in a hidden form.

2. Obesity and Adiposity

The fundamental cause of obesity is a long-term alteration in energy balance, where energy intake exceeds energy expenditure. Therefore, it is believed that AS such as sucralose and aspartame promote weight loss and weight maintenance since energy dense food and beverages are replaced with non-caloric sweeteners. In fact, cohort data demonstrated that replacing sugar sweetened beverages with artificially sweetened beverages is associated with a promotion in weight reduction(19, 20). Another animal study conducted by Mitsutomi et al. showed that aspartame has a neutral effect on body weight. 4% aspartame for 4 weeks failed to increase weight among diet-induced obese mice(16). However, other animal studies showed the opposite. In fact, aspartame and sucralose are associated with increased weight gain, adiposity, and food intake. Feijo et al. found that aspartame increased weight gain and adiposity, but unrelated to caloric intake(21). Also, Mitsutomi et al. showed that diet-induced obese mice consuming aspartame sweetened water at a dosage of 4% for 4 weeks had increased adiposity (weight remained the same) when compared with the control group. It also increased tissue triglyceride levels in the liver and skeletal muscles (increased visceral fat)(16). In addition, several types of human studies indicated as well a positive association between

AS (including sucralose and aspartame) consumption and obesity (especially central obesity). For example, Ruanpeng et al. indicated in a meta-analysis that the relative risk (RR) for obesity in people who consume sugar sweetened soda is 1.18 (95% CI, 1.10-1.27) less than RR for obesity in people who consumed AS soda(sweetened in aspartame, sucralose, or other types of AS) = 1.59(95% CI, 1.22-2.02)(18). Also, long term cohort studies demonstrated that chronic use of AS such as sucralose and aspartame in food and beverages increases BMI and abdominal obesity. AS beverages increase overweight risk for consumers who were healthy weight at baseline, and increase obesity risk for consumers who were overweight at baseline(19, 22). The weight loss resulting for AS use is only for a short term. A simple behavioral mechanism could explain the association between AS and obesity as well. In fact, AS are usually present in unhealthy ultra-processed food. When a person consumes AS in processed food he is more likely to be replacing healthy food (fruits, vegetables, pulses...) with ultra-processed food and adapting an unhealthy diet that could contribute to obesity, and metabolic diseases. Controversially, another theory says that individuals who consume AS such as aspartame and sucralose are most likely to be interested in maintaining a healthy lifestyle, monitor their weight, and consume healthy food. Thus, AS can be confounding factors and human studies that showed that aspartame and sucralose promote weight loss might be biased.

3. Gut Microbiota

The gut microbiota is implicated in the pathogenesis of chronic diseases. In fact, gut microbiota is involved in the digestion of macronutrients. The resulting metabolites can serve as epigenetic activators of gene expression, and may influence the

disease risk. Also, gut microbiota plays an important role in energy storage, intake, and expenditure. Research studies are focusing on the impact of AS on gut microbiota, to examine the harmful mechanistic effects of AS on the metabolism. One possible mechanism is that usually, the gut microbiota produces short chain fatty acids (SCFA) (butyrate, propionate, and acetate) that bind to GPCRs; therefore, they activate insulin receptor signaling, resulting in an increase in insulin sensitivity, and a decrease in insulin resistance. Additionally, the SCFA stimulate leptin release, slowing gut motility, and increasing lipogenesis. SCFA produced by gut microbiota can stimulate the decrease in insulin signaling in adipose tissue; thus, decrease fat accumulation and increase weight loss. However, unfortunately the AS modulate the gut microbiota composition (dysbiosis). In fact, studies have shown the microbiota composition of AS consumers resembles that of obese individuals. Therefore, further to the AS consumption, the composition of the gut microbiota is altered, resulting in a decrease of SCFA production, leading to an increased risk of type 2 diabetes, metabolic syndrome, and obesity. When the GPCR performs crosstalk with the sweet taste receptors (activated by AS consumption) instead of SCFA, this will result in alterations in gut motility and permeability to SCFA, ending up with metabolic and immunological abnormalities(9).

4. Glycemia and Glucose Tolerance

Excessive sugar intake affects glycemic responses. Hence, alterations in glycemia and glucose tolerance will occur, resulting finally in diabetes. AS were introduced to replace sugar, and prevent this burden. Grotz et al. stated that both HbA1C and fasting blood glucose decreased in diabetics consuming sucralose for 3

months(13, 23). Other studies have shown neutral effects of AS on glycemic response(10). Nevertheless, many human and animal interventional studies showed that sucralose and aspartame worsen glucose tolerance in both lean and obese (even in healthy and inuslin-sensitive obese) consumers. Also the negative effects of aspartame and sucralose on glucose tolerance and insulin sensitivity was shown similarly among individuals that are usually not adapted to an AS-dependent diet. It is hypothesized that the reason why some people are more prone to have glucose intolerance after AS consumption, is due to the gut microbiota(14). Additionally, prospective cohort studies in different populations, with different dietary patterns indicated that aspartame consumption does not decrease the incidence of diabetes, in fact it increases it more, when compared with sugar-sweetened beverages. The effect is more pronounced among individuals with higher adiposity(14, 24). An animal study demonstrated that even a small dose of aspartame is associated with an increased risk of glucose intolerance. In the study, rats consuming aspartame had low energy intake and gained less weight than rats on high fat diet. However, the rats in the aspartame group had higher fasting blood glucose, increased insulin intolerance, and altered gut microbiota (*fermiticus*/ *bacteroidetes* ratio), independent of body fat composition(22).

There are three mechanistic explanations for the harmful effects of AS on glycemic response. Firstly, after the consumption of AS, the sweet taste receptors get activated, as a result, insulin will be released to metabolize the sugar as expected. This physiological response becomes blunted, and fails to respond to actual sugars when consumed, because the sweet taste receptors can't offer reliable signals about what will happen next. Secondly, it is hypothesized that the aspartame is associated with an increase in SCFA propionate in the colon. This will result in an increase in

gluconeogenesis in the liver, leading to a hyperglycemia. This could also be associated with an increase in free fatty acid in the blood, visceral fat, insulin resistance, dyslipidemia, and other negative metabolic outcomes(14). Thirdly, researchers suggest that phenylalanine- the breakdown product of aspartame inhibits intestinal alkaline phosphatase, an enzyme that is associated with a reduced risk of metabolic syndrome (it regulates pH of intestine; thus, affects gut microbiota, absorption of lipids and other nutrients)(14).

5. Insulin Sensitivity

Insulin is an anabolic hormone that plays multiple crucial roles in the body such as regulation of blood glucose and appetite. Sugar consumption disrupts insulin. Hence, replacement of sugar with AS will supposedly serve individuals that enjoy the sweet taste. Many studies reported that there isn't a significant association between aspartame and sucralose usage and modifications in insulin sensitivity and insulin concentration(25). For instance, a randomized crossover trial indicated that 425mg/day of aspartame and 136mg/day of sucralose for 2 weeks failed to show any effects on insulin sensitivity (measured by HOMA-IR, HOMA-%B, and HIMA-%S)(25). In contrast, other research work proved that both acute and chronic use of sucralose have detrimental effects on insulin, insulin sensitivity and response(25). In an animal study, Mitsutomi et al. indicated that although in case of sugar consumption, the blood glucose levels seemed to be higher than in case of aspartame consumption; however, the insulin level was higher in the aspartame group when compared with the sucrose group. Also, the glucose tolerance test indicated that glucose loading in the aspartame group increased blood glucose significantly more than the control group. This indicates that

aspartame consumption resulted in an increase in tissue triglyceride (visceral fat) and an increase in insulin resistance(16). Another study demonstrated that after consumption of 9 grams of Canderel (191.70 mg aspartame), blood glucose did not change significantly, but insulin increased(26). Therefore, artificial sweeteners should not be used among obese individuals and diabetics because they don't help in controlling glycaemia, on the contrary they provide more harms than benefit(16). A possible explanation for the harmful effects of AS on insulin was linked to the detrimental effect of AS on gut microbiota. When the gut microbiota composition changes, the body produces a low-grade inflammatory state (metabolic endotoxemia), resulting in insulin resistance(9).

6. Blood Lipids

Dyslipidemia is a key factor for metabolic syndrome that can increase the risk of chronic diseases and obesity. Some studies showed that AS consumption is a solution to prevent and treat metabolic syndrome and related diseases because they do not increase serum triglyceride (TG), when compared with sucrose consumption(16). However, other studies argued and showed that AS, particularly aspartame are lipogenic. In fact, chronic intake of aspartame significantly increased lipid peroxidation products in the liver and other organs (kidneys and brain)(27). This shows that aspartame consumption results in liver, kidney, and brain damages(28). When AS are consumed, sweet taste receptors are activated in the tongue and the gastrointestinal tract. Insulin is secreted as a response to the sweetness because the body theorizes that sweet taste is associated with sugar intake and it needs to be metabolized by insulin. Surprisingly, insulin doesn't find a sugar to metabolize it. Chronic AS consumption exhausts the pancreatic β cells, and results in insulin resistance despite the

normoglycemia. Therefore, alteration in fatty acid oxidation will occur and it will lead to dyslipidemia characterized by an increase in TG, increase in small dense low-density lipoprotein (LDL), and decrease in high-density lipoprotein (HDL)(29). Also, AS increase central obesity, visceral fat, and tissue triglyceride levels in the liver and skeletal muscle that could explain the mechanism of AS association with dyslipidemia(16, 27).

7. Appetite

Appetite is the psychological desire for foods or beverages. Many factors influence appetite, including sensory responses to the tastes of food. According to studies supported by FDA, aspartame and sucralose has neutral effects on appetite(10). Other studies do not support these findings. For example, AS exposure was proven to increase appetite, hunger, food consumption, and cravings(14, 19). In fact, once AS activate the taste receptors in the tongue, the food reward pathway gets activated only partially, and fail to activate the post-ingestion pathway because of the lack of caloric energy. Changes in these pathways ultimately contribute to increased appetite, food craving, and caloric consumption(14). Furthermore, new data from both human and animal models provided convincing evidence that AS are associated with glucagon-like peptide-1 (GLP-1). GLP-1 is an anorexigenic gut hormone secreted by the enterocytes in response to the presence of nutrients in the small intestine. The GLP-1 receptor is expressed in the pancreatic islets, in hypothalamus and brain. Also, GLP-1 activation is coupled with insulin secretion. AS consumption was associated with a disruption in GLP-1 secretion, resulting in alterations in the hunger-satiety cycle, and affecting insulin response, appetite, and energy intake(9, 30). Moreover, animal studies showed

that AS have negative effects on appetite by affecting leptin. Leptin is also an anorexigenic hormone secreted by adipocytes. It has crucial metabolic roles, most importantly it regulates the energy homeostasis by controlling appetite, food intake, and weight. It has receptors in the brain that control appetite and regulates the energy homeostasis. When leptin gets activated, it stimulates further anorexigenic compounds involved in the energy homeostasis (such as Proopiomelanocortin (POMC))(31). Unfortunately, AS has harmful effects on leptin. In fact, animal studies have shown that elevated levels of aspartate (a major constituent of aspartame) show a detrimental effect on the neurons present in the arcuate nucleus of the hypothalamus, which is a site for leptin to reduce food intake and promote appetite(19). Also, AS contributed to leptin resistance that altered the energy homeostasis system(16).

8. Pregnancy

Pregnancy is a critical period were mothers should be cautious to prevent any exposure that could irreversibly affect the development of the fetus or embryo. According to FDA, AS are safe to use by everyone. However, AS and their breakdown products cross the placenta. It is not clear whether AS have any teratogenic effect. Recent data showed that maternal consumption of artificial sweetened beverages (mainly as aspartame used in carbonated beverages) during pregnancy is associated with an increased risk of preterm delivery, a greater infant BMI. In fact, the earlier the exposure of aspartame, the more profound the damage. After conducting rodents' studies, it is hypothesized that aspartame is neurotoxic. Intra uterine exposure is associated with leptin resistance and central obesity, because aspartame damages neurons in the arcuate nucleus of the hypothalamus, that is a key site for leptin(19).

Ergo, it is questionable whether it is safe for pregnant women to consume AS; FDA needs to reconsider this matter.

Unfortunately, AS sweeteners exist in hidden forms in food, beverages, and other products. Therefore, it is very challenging to control and assess the exposure of AS among humans in order to evaluate the side effects. Thus, the outcome yielding from human studies can be inconsistent; interventional animal studies are more controlled. The objective of this rodent study is to assess the metabolic effects of usual amounts of aspartame and sucralose (commonly used AS) in food and/or water. The study mimics real life situations since sucralose and aspartame are present in a variety of food and beverages.

CHAPTER III

MATERIALS AND METHODS

A. Animal Care

Mature Sprague Dawley male rats (n=48) were obtained from the Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut-Lebanon. Rats were housed in a light, temperature, and humidity-controlled room (reverse light cycle: 12-h light/12-h dark cycle reverse; lights are off at 10 am) for approximately 8 weeks (one rat per cage). Rats had free access to food (ad libitum), water was switched every 12-h for the group with sweetened water (sweet water when lights are off because rats are nocturnal). All rats were treated according to the guidelines of IACUC of the American University of Beirut, and our study was approved by this committee.

B. Experimental Design

One week of adaptation was performed to familiarize the rats with the environment. After that, rats were divided into 7 interventional groups:

- Group 1(Control Group): includes diet with starch and regular unsweetened water (n=6)
- Group 2 (Asp. Diet+ Regular Water): includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water (n=7)
- Group 3 (Scl. Diet+ Regular Water): includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water (n=7)

- Group 4 (Regular Starch Diet+ Asp. Water): contains diet with starch and 0.25g/500g aspartame-sweetened water (n=7)
- Group 5 (Asp. Diet+ Asp. Water): consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water (n=7)
- Group 6 (Regular Starch Diet+ Scl. Water): contains diet with starch and 0.08g/500g sucralose-sweetened water (n=7)
- Group 7 (Scl. Diet+ Scl. Water): includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water (n=7)

The diet offered is prepared by the researchers. It has the same composition of the AIN-93G diet(32); however, sucrose was replaced with starch in order to assess if AS are healthy substitutes of sucrose. 0.5g/kg aspartame was added to the food of Group 2 (Asp. Diet+ Regular Water) and Group 5 (Asp. Diet+ Asp. Water), corresponding to the same power of sweetness of sucrose. 0.16g/kg sucralose was added to the food of groups Group 3 (Scl. Diet+ Regular Water) and Group 7 (Scl. Diet+ Scl. Water), in order to correspond to the same power of sweetness of sucrose (Table 1). Also, 0.25g/ml aspartame was added to the water Group 4 (Regular Starch Diet+ Asp. Water) and Group 5 (Asp. Diet+ Asp. Water). 0.08g/ml of sucralose was added to the water of Group 3 (Scl. Diet+ Regular Water) and Group 7 (Scl. Diet+ Scl. Water), in order to correspond to the same power of sweetness of sucrose. Water (500ml) was changed and refilled once per week, and food was changed and refilled twice per week. The sweetened food was available all the time (ad libitum feeding), and the water was switched every 12 h since rats are nocturnal (sweet water when lights were turned off).

Table 1: Dietary composition of the prepared experimental diets based on the composition of the AIN-93G diet

Diets				
Ingredients	Quantities (g/Kg) in the AIN-93G diet	Quantities (g/Kg) in the Starch Diet	Quantities (g/Kg) in the Aspartame Diet	Quantities (g/Kg) in the Sucralose Diet
Casein	200	200	200	200
L-Methionine	3	3	3	3
Starch	532	632	632	632
Sucrose	100	0	0	0
Aspartame	0	0	0.5	0
Sucralose	0	0	0	0.16
Oil	70	70	70	70
Cellulose	50	50	50	50
Mineral Mix	35	35	35	35
Vitamin Mix[1]	10	10	10	10
Total Weight[2]	1000	1000	1000	1000

[1] *Mineral mix (AIN-93G, used at 35 g/kg of diet, obtained from Dyets inc.)*
[2] *Vitamin mix (AIN-93VX, used at 10 g/kg of diet, obtained from Dyets inc.)*

1. Assessment of Food and Fluid Intake

Food intake was assessed by measuring twice/week the food consumed, for 8 interventional weeks, taking into consideration the food spillage. As for the water intake, it was assessed by measuring once/week the water consumed (both sweetened and unsweetened), for 8 interventional weeks.

2. Body Weight and Body Composition

Body weight (g) was determined on weekly basis, using a calibrated digital weighing machine. Body composition (lean mass (g) and body fat mass(g)) of rats was also measured weekly via the machine NMR minispec (LF110 BCA analyzer, Brucker, MA, USA).

3. Sacrifice

Prior to the 2 days of sacrifice, preparations have been made. **(Appendix 1)*

On the day of sacrifice:

After 8 weeks of intervention, overnight fasted rats were put inside a container containing cotton and filled with inhalation anesthetic isoflurane (Forane®, 134 Abbott, Berks, UK). Once the rat is anesthetized, fasting weight was measured via a digital scale. The head of the rat was placed inside an anesthetic mask that contains isoflurane, rat was dissected using scissors. Blood was collected slowly from the superior vena cava, because it is a big vein which makes it easier to collect blood; thus, the output is more specific. After that, the rat was sacrificed by severing his heart. Heart, liver, kidneys, and epididymal adipose tissues were immediately excised, dried from blood (because it can affect the significance of the organ weight), weighed, frozen in liquid

nitrogen, and stored at -80°C. A piece of the liver was placed in a chloroform tube that preserves the hepatic tissues, in order to perform a histological analysis. The tubes were stored at the room temperature in a labelled box. Blood samples were centrifuged at 2200 g (3°C) for 15 min, and aliquots of plasma were collected and stored at -80°C until analyzed. Needles were thrown in a sharp container, and the waste was thrown in a yellow labeled biohazardous bag.

4. Plasma Analysis

a. Fasting Insulin

Insulin was analyzed using the "enzyme-linked immunosorbent assay" (ELISA) immunoassay technique. The ELISA kits were purchased from "Merck Millipore", EZRMI-13K. ***(Appendix 2)*

b. Serum Metabolic Markers (glucose, lipid profile (TG, total cholesterol (TC), HDL, albumin, creatinine (crea), and blood urea nitrogen (BUN)

A calibrated Vitros 350 machine was used to perform serum analysis of glucose, TG, TC, HDL, albumin, crea, and BUN. ****(Appendix 3)*

C. Statistical Analysis

Statistical analysis was performed using the software program "Statistical Package for the Social Sciences-21" (SPSS-21). Data was analyzed by a one-way or two-way analysis of variance (ANOVA) tests depending whether time was a factor (two-way ANOVA), or not (one-way ANOVA).

CHAPTER IV

RESULTS

A. Food and Fluid Intake

1. Food intake

All values are expressed as mean ± standard deviation (SD). Significance is found with P-value < 0.05. The food intake was assessed twice per week for 7 interventional weeks. Data of food intake is presented as average weekly food intake (grams per week) among the 7 groups over the 7 weeks' interventional period (table 2 and figure 1). A two-way analysis of variance (ANOVA) test is performed, with the food intake being the dependent variable, and both the interventional group and time are the independent variables (factors).

The data shows that group 6 (Regular Starch Diet+ Scl. Water) has the lowest mean of food intake, and the group 4 (Regular Starch Diet+ Asp. Water) has the highest mean of food intake (table 2).

There isn't a significant difference between the group 1 (Control Group), and the other interventional groups of rats fed aspartame or sucralose. But there is a significant difference between the interventional groups. The average food intake is significantly lower in group 6 (Regular Starch Diet+ Scl. Water) than the group 4 (Regular Starch Diet+ Asp. Water) *(P= 0.003),* 5(Asp. Diet+ Asp. Water) *(P=0.009),* and 7 (Scl. Diet+ Scl. Water) *(P=0.026).*

2. Fluid Intake

All values are expressed as mean ± SD. Significance is found with P-value <0.05. The fluid intake of both sweet and regular water was assessed once per week for 7 interventional weeks. To calculate the fluid intake, the weight of empty water bottles (both containing sweetened and unsweetened fluid) was measured, and the value was subtracted from the weight of the bottle full measured in the previous week.

All the interventional groups were exposed to regular unsweetened water. Group 1(Control Group), Group 2 (Asp. Diet+ Regular Water), and Group 3 (Scl. Diet+ Regular Water) were exposed to regular water only, whereas Group 4 (Regular Starch Diet+ Asp. Water), Group 5 (Asp. Diet+ Asp. Water), Group 6 (Regular Starch Diet+ Scl. Water), and Group 7 (Scl. Diet+ Scl. Water) were exposed to both sweetened water and regular water, switched every 12 hours.

- Concerning the regular fluid intake, data is presented as average of weekly regular unsweetened fluid intake (grams per week) among the 7 groups over the 7 weeks' interventional period (table 3, and figure 2). A two-way ANOVA test is performed, regular unsweetened fluid intake was the dependent variable, and both the interventional group and time were the independent variables.

Among the groups only exposed to regular fluid, the data indicates that the highest mean of regular fluid intake is among Group 1(Control Group), and the lowest is among Group 2 (Asp. Diet+ Regular Water) (figure 3). Also, Group 1(Control Group) has a significantly higher mean of regular fluid intake than Group 2 (Asp. Diet+ Regular Water) *(P<0.0002).*

Among the groups exposed to both regular and sweetened fluid, the data indicated that the highest percentage regular fluid intake is among is for 6 (Regular

Starch Diet+ Scl. Water) (56%), and the lowest is for Group 5 (Asp. Diet+ Asp. Water) (52%) (figure 4). However, there isn't a statistically significant difference in regular unsweetened fluid intake between all the groups.

- As for the sweetened-fluid intake, the data is presented (in grams per week) as average weekly consumption of aspartame-sweetened water among the Group 4 (Regular Starch Diet+ Asp. Water) and Group 5 (Asp. Diet+ Asp. Water), and sucralose- sweetened water among the Group 6 (Regular Starch Diet+ Scl. Water), and Group 7 (Scl. Diet+ Scl. Water) over the 7 weeks' interventional period (table 4 and figure 5). Percentage of sweetened fluid intake was calculated. A two-way ANOVA test is performed, percentage sweetened fluid intake was the dependent variable, and both the interventional group and time were the independent variables. The results show that the highest percentage of sweetened-fluid consumption is among Group 5 (Asp. Diet+ Asp. Water) (48.5%), and the lowest is among Group 6 (Regular Starch Diet+ Scl. Water) (44.6%) (figure 6). There isn't a statistical significant difference of percentage sweetened fluid intake among the groups exposed to aspartame-/sucralose-sweetened water.

- Regarding the total fluid intake, it was calculated by summing the sweet fluid consumed and the regular unsweetened fluid consumed in case of the groups exposed to aspartame-/ sucralose- sweetened fluid (Group 4 (Regular Starch Diet+ Asp. Water), Group 5 (Asp. Diet+ Asp. Water), Group 6 (Regular Starch Diet+ Scl. Water), and Group 7 (Scl. Diet+ Scl. Water). In case of the Group 1(Control Group), Group 2 (Asp. Diet+ Regular Water), and Group 3 (Scl. Diet+ Regular Water), only exposed to regular water, the regular water consumed was considered as total fluid intake. Data is presented as mean of weekly total fluid intake (grams per week) among the 7 groups

over the 7 weeks' interventional period (table 5 and figure 8). A two-way ANOVA statistical test is completed; the data indicates that the highest mean of total fluid intake is among the Group 1(Control Group), and the lowest mean belongs to Group 2 (Asp. Diet+ Regular Water) (figure9). The mean of total fluid consumption is significantly higher among Group 1(Control Group) than both Group 2 (Asp. Diet+ Regular Water) *(P=0.012)*, and Group 6 (Regular Starch Diet+ Scl. Water) *(P=0.030)*.

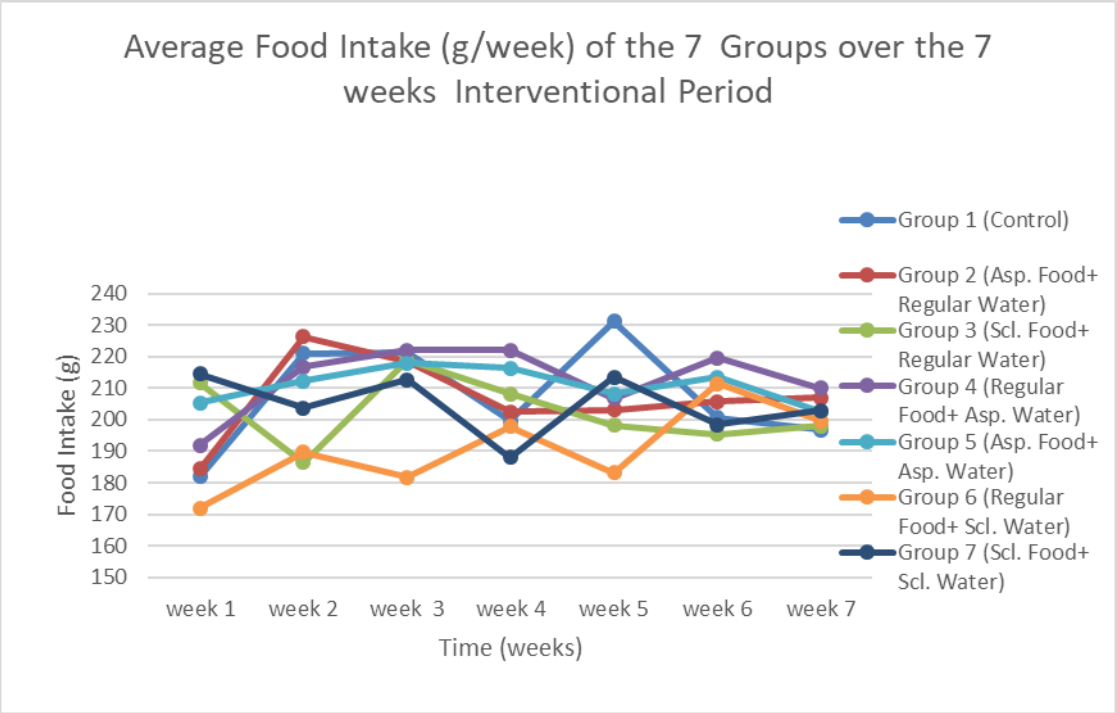
Table 2: Average of food intake in grams per week of the seven interventional groups, during the 8 weeks interventional period Group

	Group 1 Control n=6	Group 2 Asp. Diet+ Regular Water n=7	Group 3 Scl. Diet+ Regular Water n=7	Group 4 Regular Starch Diet+ Asp. Water n=7	Group 5 Asp. Diet+ Asp. Water n=7	Group 6 Regular Starch Diet+ Scl. Water n=7	Group 7 Scl. Diet+ Scl. Water n=7
Week 1	181.9±11.0	184.3±10.2	211.7±10.2	192.0±10.2	205.4±10.2	172.0±10.2	214.6±10.2
Week 2	220.9±11.0	226.3±10.2	186.3±10.2	216.7±12.1	212.3±10.2	189.6±10.2	203.6±10.2
Week 3	221.4±11.0	218.3±10.2	218.9±10.2	222.0±10.2	218.0±10.2	181.8±12.1	212.6±10.2
Week 4	199.6±12.1	202.5±10.2	208.2±10.2	222.0±10.2	216.4±10.2	197.7±10.2	219.3±11.0
Week 5	231.2±11.0	203.1±10.2	198.1±10.2	206.9±10.2	208.2±12.1	183.2±11.0	213.5±10.2
Week 6	200.7±11.0	205.7±10.2	195.4±10.2	219.6±10.2	213.5±10.2	211.5±10.2	198.3±10.2
Week 7	196.9±11.0	207.0±10.2	197.9±10.2	210.0±10.2	202.4±10.2	199.6±10.2	202.9±10.2
Average Food Intake (g/ week)	207.7±33.9	206.8±24.2	202.4±31.7	212.5±29.1 [a]	211.0±18.8 [b]	191.3±28.2 [a b c]	209.0±24.3 [c]

[a b c] *statistically significant difference exists*

Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. All values are expressed as Mean ±SD (Standard Deviation). Two-way ANOVA test is performed with food intake being the dependent variable and both the interventional group and time being the factors. Significance is found with P-value<0.05.

Figure 1: Variation of the Mean Food Intake in Grams per Week of the Seven Groups over the 7 weeks interventional period



Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water.

All values are expressed as mean. To assess the effect of time, interventional group, and interaction time*group on food intake, a two-way ANOVA test is performed with food intake being the dependent variable and both the interventional group and time being the factors. Significance is found with P-value<0.05.

Time has a significant effect on food intake (*P= 0.036*). On week 3, food intake started to increase significantly compared to the beginning of the study (*P= 0.009*). Also, the interventional group has a significant effect on food intake (*P= 0.003*). Mean food intake of Group 6 (Regular Starch Diet+ Scl. Water) is significantly lower than Group 4 (Regular Starch Diet+ Asp. Water) *(P=0.003)*, Group 5 (Asp. Diet+ Asp. Water) *(P=0.009)*, and Group 7 (Scl. Diet+ Scl. Water) *(P=0.026)*. But the interaction time*group does not have a significant effect on food intake (P= 0.321).

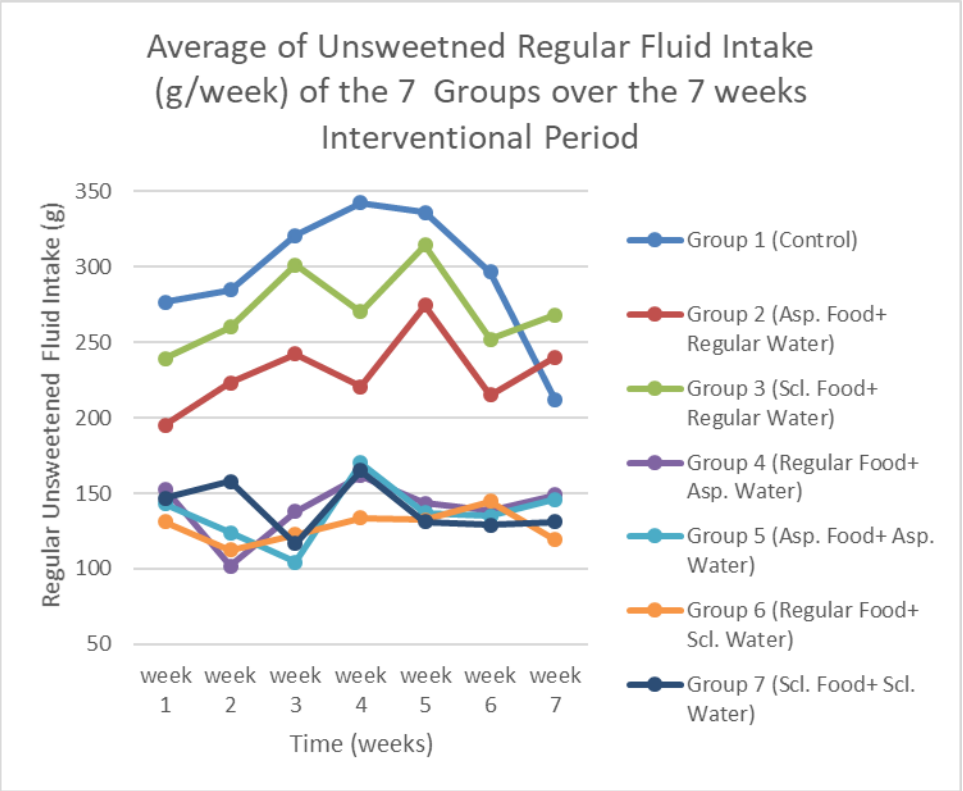
Table 3: Mean of regular unsweetened fluid intake in grams per week among the seven groups over the seven weeks interventional period

	Group 1 (Control) n=6	Group 2 (Asp. Diet+ Regular Water) n=7	Group 3 (Scl. Diet+ Regular Water) n=7	Group 4 (Regular Starch Diet+ Asp. Water) n=7	Group 5 (Asp. Diet+ Asp. Water) n=7	Group 6 (Scl. Water+ Regular Starch Diet) n=7	Group 7 (Scl. Diet+ Scl. Water) n=7
Week 1	276.6±114.9	195.2±32.6	239.4±60.6	152.6±33.2	143.0±43.4	131.2±15.3	146.9±41.6
Week 2	284.9±132.3	223.3±44.9	260.4±83.3	101.8±25.8	124.0±34.7	112.5±36.9	158.1±63.7
Week 3	320.7±152.5	242.3±32.4	301.4±100.7	138.1±44.2	104.3±38.8	122.7±41.4	116.6±35.2
Week 4	342.4±186.9	220.8±31.0	270.6±98.4	161.7±41.5	170.1±37.6	133.8±27.7	165.0±45.4
Week 5	336.3±162.8	274.5±41.7	314.7±88.7	143.5±57.1	137.1±25.2	132.4±49.0	131.2±43.8
Week 6	296.5±137.5	215.2±42.5	252.3±96.8	138.2±57.8	134.9±24.8	144.8±60.3	129.2±55.0
Week 7	211.6±92.9	240.4±56.7	268.2±70.6	148.9±52.2	145.9±17.1	119.4±45.8	131.2±44.3
Average Regular Fluid Intake (g/ week	295.6±138.5 [a]	230.3±45.1 [a]	272.4±85.2	140.7±46.5	137.0±36.1	128.1±40.3	139.7±47.6

[a] *statistically significant difference exists*

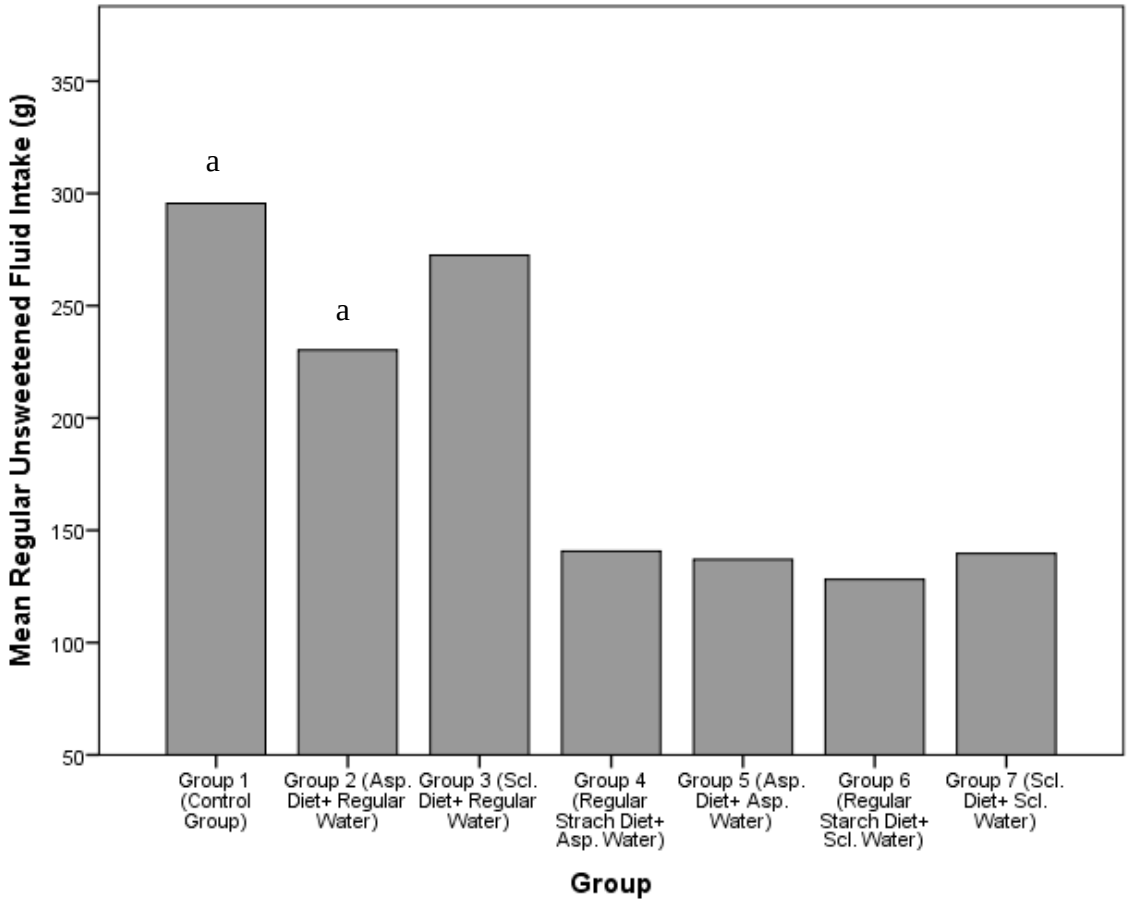
Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. All values are expressed as Mean ±SD (Standard Deviation). Two-way ANOVA test is performed with regular unsweetened fluid intake being the dependent variable and both the interventional group and time being the factors. Significance is found with P-value<0.05.

Figure 2: Mean of regular unsweetened fluid intake in grams per week among the seven groups over the seven weeks interventional period



Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water

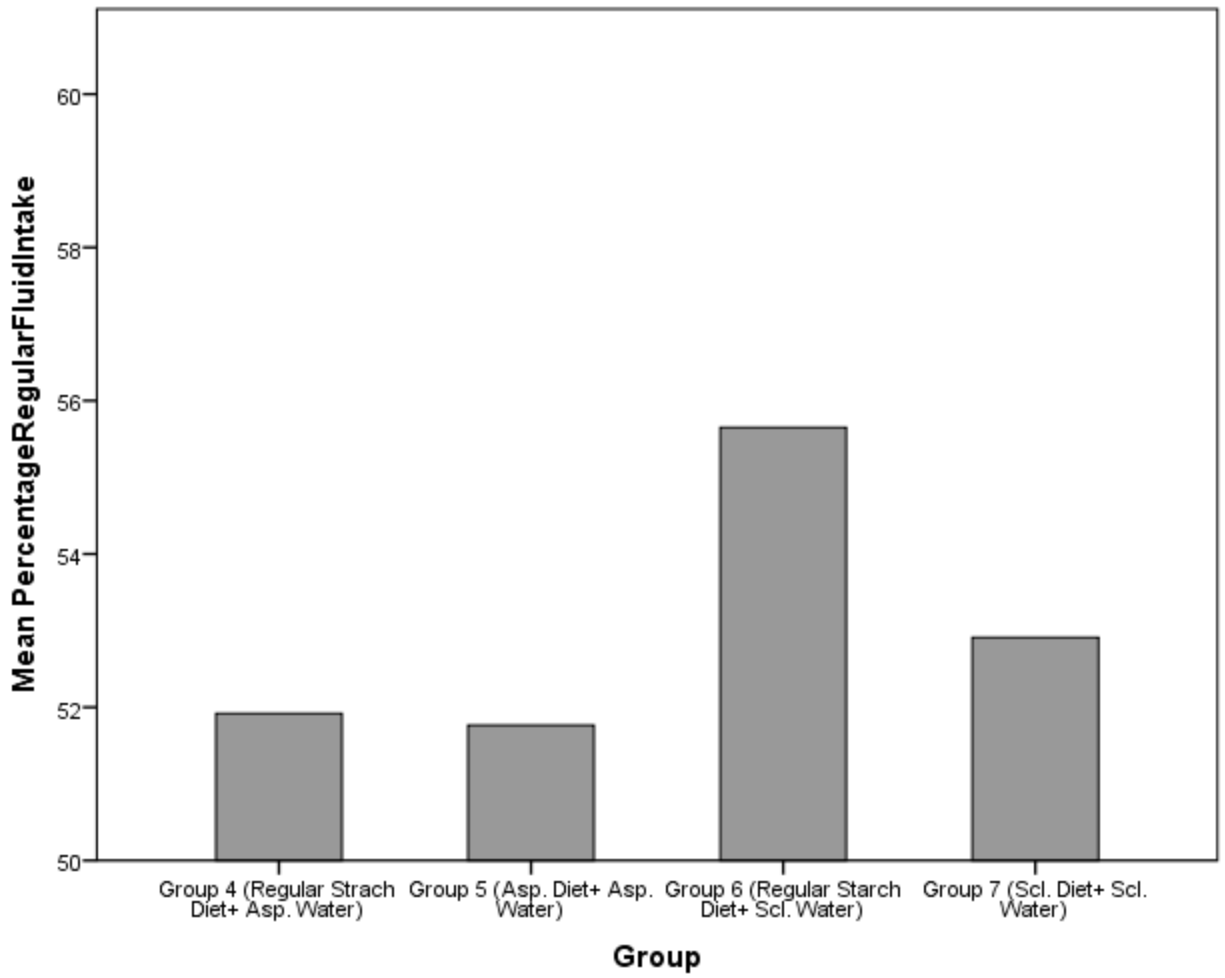
Figure 3: Mean of regular unsweetened fluid intake in grams the seven groups



[a] *statistically significant difference exists.*

Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. All values are expressed as mean. Two-way ANOVA test is performed with regular unsweetened fluid intake being the dependent variable and both the interventional group and time being the factors. Significance is found with P-value<0.05.

Figure 4: Mean percentage of regular unsweetened fluid intake among the interventional groups exposed to sweet water



Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water.

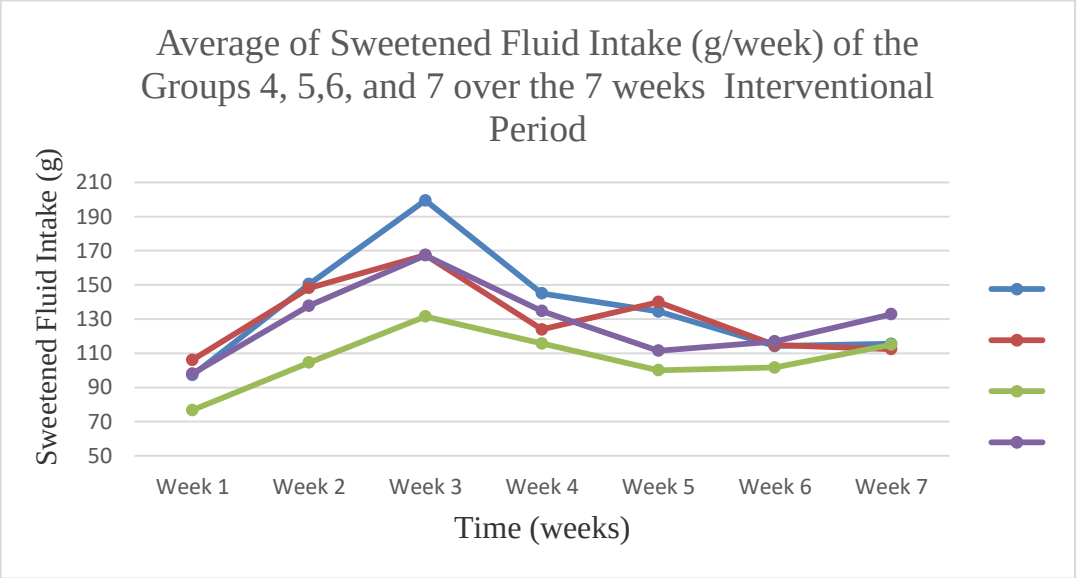
Table 4: Mean of sweetened fluid intake among the interventional groups exposed to sweet water over the seven weeks interventional period

	Group 4 (Regular Starch Diet+ Asp. Water) n=7	Group 5 (Asp. Diet+ Asp. Water) n=7	Group 6 (Scl. Water+ Regular Starch Diet) n=7	Group 7 (Scl. Diet+ Scl. Water) n=7
Week 1	97.3±15.7	106.2±17.0	76.7±33.0	98.1±27.6
Week 2	150.5±42.9	148.2±50.0	104.6±48.1	137.9±84.3
Week 3	199.4±79.7	167.5±67.4	131.5±54.4	167.3±115.5
Week 4	145.0±70.7	123.9±27.1	115.7±85.1	134.7±76.3
Week 5	134.4±75.2	140.0±59.4	100.1±60.3	111.5±34.6
Week 6	114.2±49.5	114.7±22.7	101.7±38.0	116.9±51.1
Week 7	115.5±53.7	112.5±18.2	115.1±42.3	132.8±44.1
Average Sweetened Fluid Intake (g/ week)	136.6±63.5 [a]	130.4±43.7	106.5±53.0 [a]	128.5±67.3

[a] *statistically significant difference exists.*

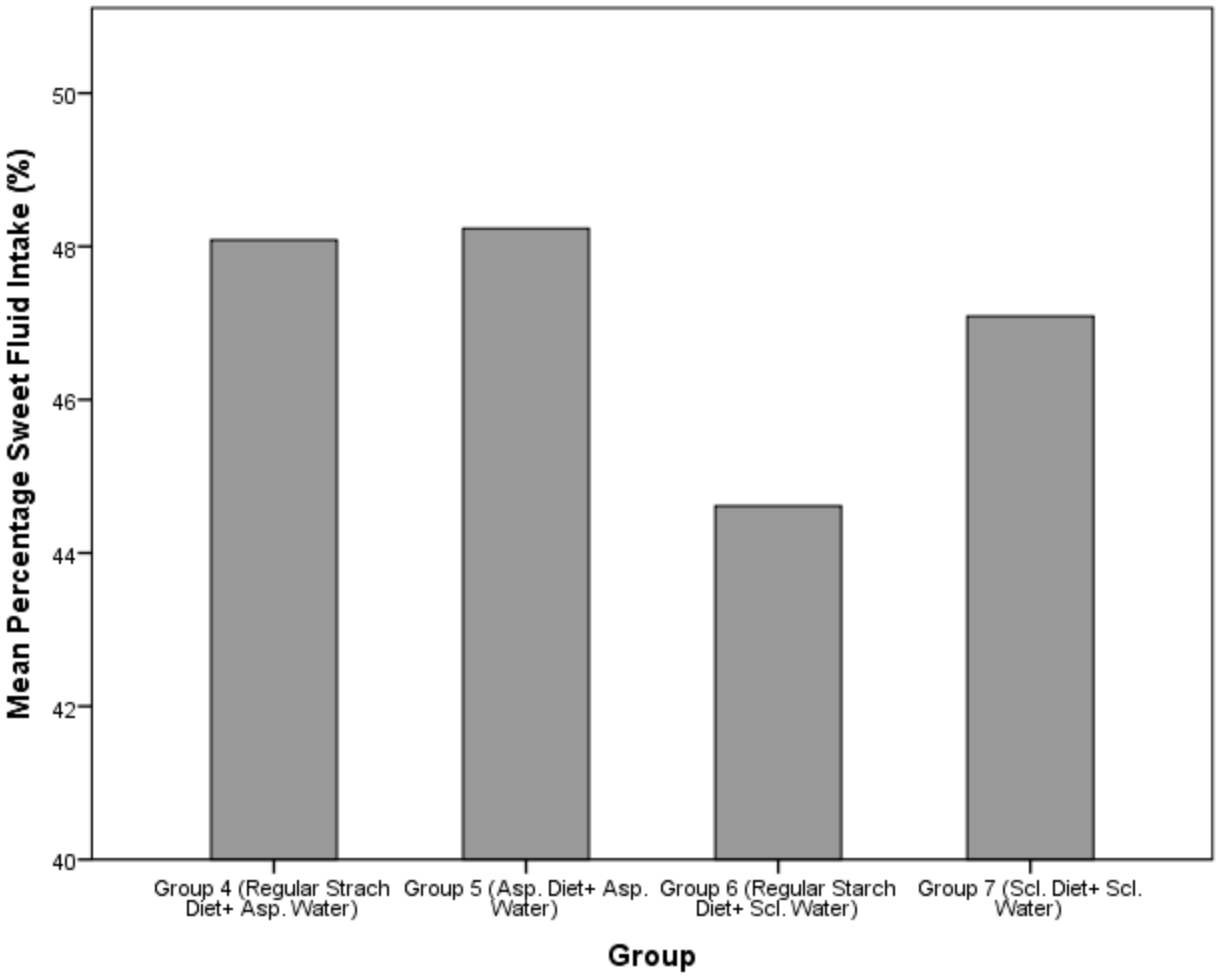
Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame-sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water.
All values are expressed as Mean ±SD (Standard Deviation). Two-way ANOVA test is performed with sweetened fluid intake being the dependent variable and both the interventional group and time being the factors. Significance is found with P-value<0.05.

Figure 5: Mean of sweetened fluid intake among the interventional groups exposed to sweet water over the seven weeks interventional period



Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame-sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water.

Figure 6: mean percentage of sweetened fluid intake among the interventional groups exposed to sweet water



Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame-sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water.

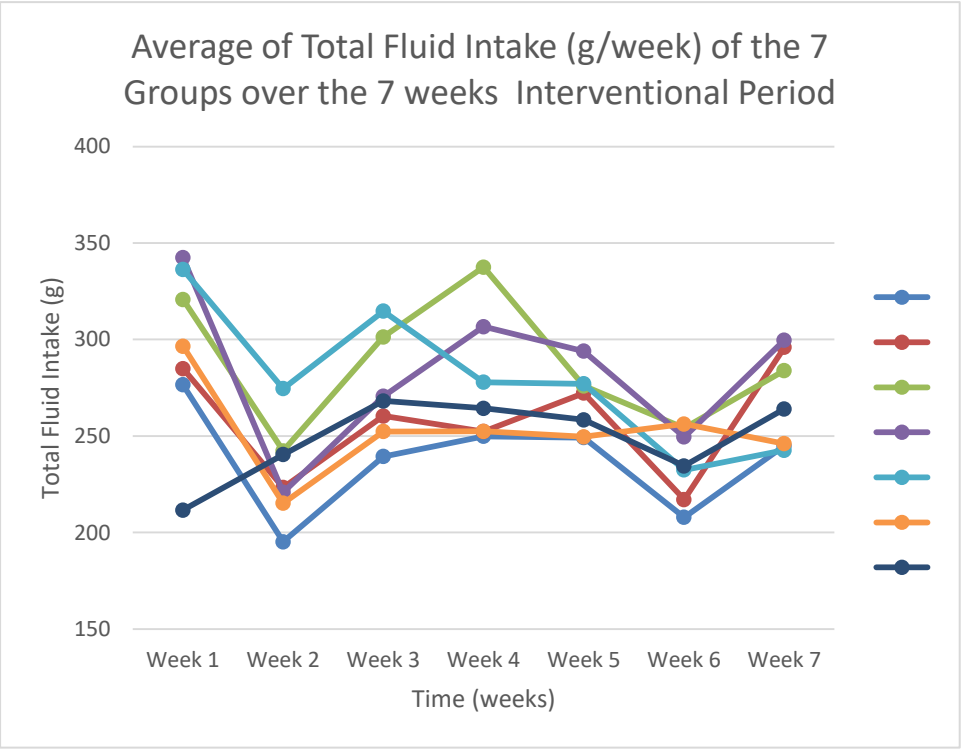
Table 5: Mean of total fluid intake in grams per week among the seven groups over the seven weeks interventional period

	Group 1 (Control) n=6	Group 2 (Asp. Diet+ Regular Water) n=7	Group 3 (Scl. Diet+ Regular Water) n=7	Group 4 (Regular Starch Diet+ Asp. Water) n=7	Group 5 (Asp. Diet+ Asp. Water) n=7	Group 6 (Scl. Water+ Regular Starch Diet) n=7	Group 7 (Scl. Diet+ Scl. Water) n=7
Week 1	276.6±114.9	195.2±32.6	239.4±60.6	249.9±35.4	249.2±54.9	207.9±42.5	245.0±55.6
Week 2	284.9±132.3	223.3±44.9	260.4±83.3	252.3±50.4	272.2±42.6	217.1±61.8	295.9±139.2
Week 3	320.7±152.5	242.3±32.4	301.4±100.7	337.5±94.1	276.0±88.1	254.2±59.1	283.9±133.0
Week 4	342.4±186.9	220.8±31.0	270.6±98.3	306.7±110.4	294.0±53.7	249.5±99.2	299.7±112.2
Week 5	336.3±162.8	274.5±41.7	314.7±88.7	277.9±127.2	277.1±78.6	232.5±104.8	242.7±61.8
Week 6	296.5±137.5	215.2±42.5	252.3±96.8	252.5±106.1	249.6±37.8	256.2±132.3	246.1±84.6
Week 7	211.6±92.9	240.3±56.7	268.1±70.6	264.4±92.2	258.4±30.1	234.5±73.2	264.0±65.3
Average Total Fluid Intake (g/ week	295.6±138.5 [a b]	230.3±45.1 [a]	272.5±85.2	277.3±92.5	267.9±56.0	235.83.1±83.1 [b]	268.2±95.1

[a b] *statistically significant difference exists between the groups*

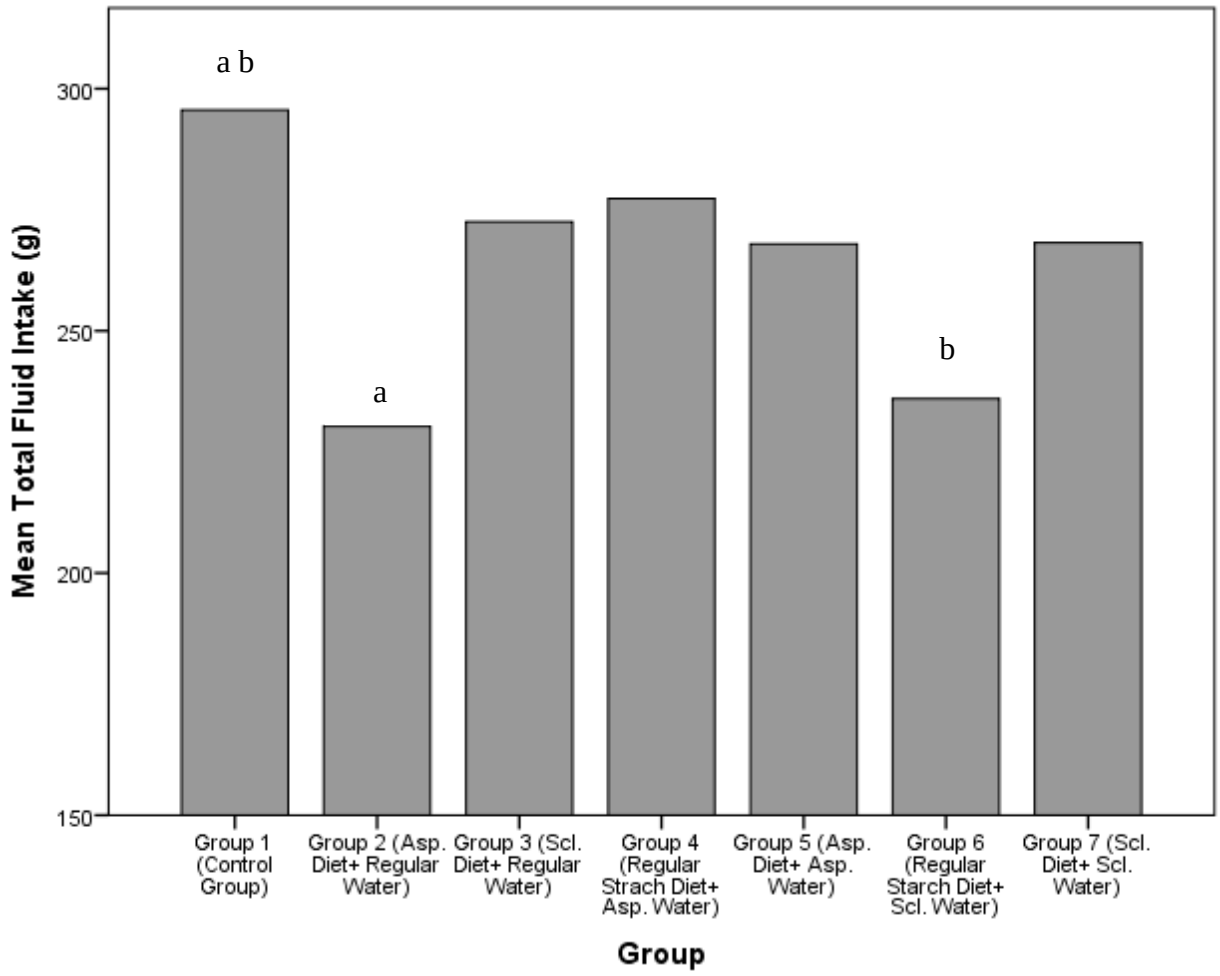
Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water.
All values are expressed as Mean ±SD (Standard Deviation). Two-way ANOVA test is performed total fluid intake being the dependent variable and both the interventional group and time being the factors. Significance is found with P-value<0.05.

Figure 7: Evolution of the mean total fluid intake in grams per week among the seven groups over the seven weeks interventional period



Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water.

Figure 8: : Mean of total fluid intake in grams among the seven groups



[a b] *statically significant difference exists between the groups*

Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water.
All values are expressed as mean. Two-way ANOVA test is performed with total fluid intake being the dependent variable and both the interventional group and time being the factors. Significance is found with P-value<0.05.

B. Body Weight and Weight Change

Data is presented as Mean ±SD. Significance is found at P-value< 0.05. The mean body weight of the rats at baseline was equal in all the seven groups (table 6). The body weight was measured weekly during the seven weeks' interventional period. Data is presented as mean body weight (g/week) for each group over seven weeks (table 6 and figure 9). It indicates that the highest mean of body weight is for Group 5 (Asp. Diet+ Asp. Water), and the lowest mean is for Group 1(Control Group). A two-way ANOVA test was performed, body weight was the dependent variable, and both the interventional group and time were the independent variables. The test revealed that the Group 5 (Asp. Diet+ Asp. Water) has a significantly higher mean body weight than Group 1(Control Group) *(P=0.034),* and a close to significant higher mean of body weight than Group 6 (Regular Starch Diet+ Scl. Water) *(P=0.056).*

Final body weight was measured on the last week of intervention. The data indicates that the highest mean of final body weight is among Group 5 (Asp. Diet+ Asp. Water), and the lowest mean is among Group 4 (Regular Starch Diet+ Asp. Water)(table 6 and figure 9). However, the one-way ANOVA test showed that there isn't a statistical significant difference of final body weight between the groups.

The weekly weight change was calculated by subtracting the weekly body weight from the baseline body weight (table 7 and figure 10). Group 5 (Asp. Diet+ Asp. Water), and Group 1(Control Group) has the lowest mean. A two-way ANOVA test was performed, weight change was the dependent variable, and both the interventional group and time were the independent variables. The results show that the weight change among Group 5 (Asp. Diet+ Asp. Water) is significantly higher

than Group 1(Control Group) *(P=0.001)*, Group 3 (Scl. Diet+ Regular Water) *(P=0.021)*, Group 4 (Regular Starch Diet+ Asp. Water) *(P=0.008)*, and Group 6 (Regular Starch Diet+ Scl. Water) *(P=0.001)*.

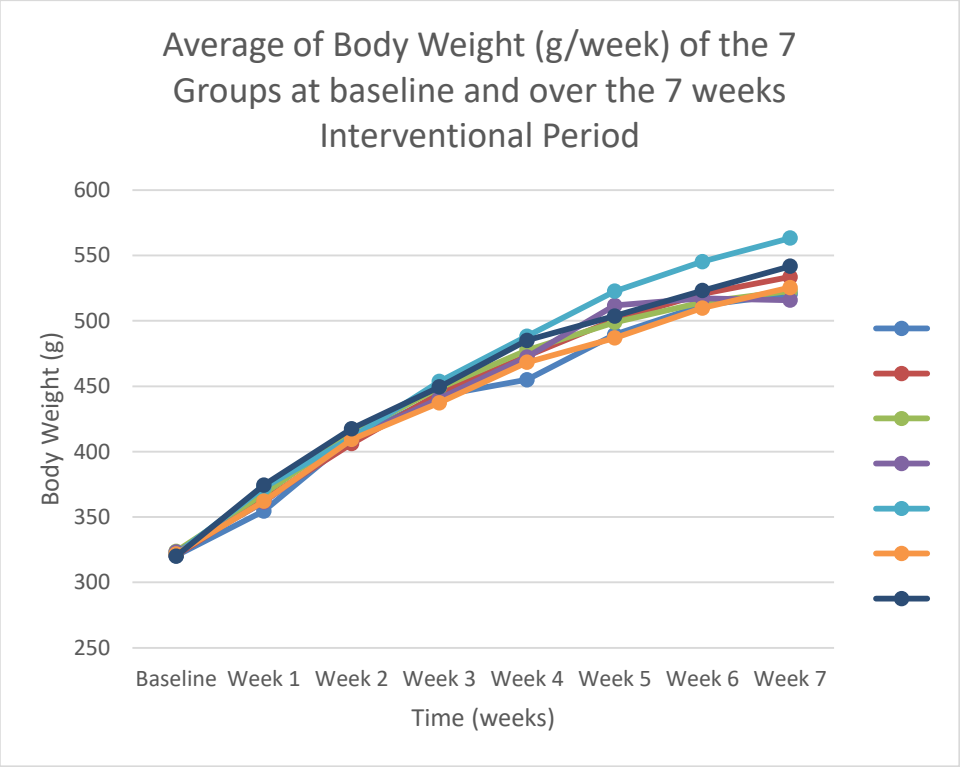
Table 6: Mean of body weight in grams of the seven groups at baseline and over the seven weeks interventional period

	Group 1 (Control) n=6	Group 2 (Asp. Diet+ Regular Water) n=7	Group 3 (Scl. Diet+ Regular Water) n=7	Group 4 (Regular Starch Diet+ Asp. Water) n=7	Group 5 (Asp. Diet+ Asp. Water) n=7	Group 6 (Scl. Water+ Regular Starch Diet) n=7	Group 7 (Scl. Diet+ Scl. Water) n=7
Baseline	320.5± 24.3	320.7± 17.1	323.8± 24.2	323.2± 24.3	321.6± 26.4	321.9± 26.7	320.2± 26.9
Week 1	354.7± 27.5	363.6± 17.2	367.9± 21.5	361.5± 30.7	372.5± 24.9	362.1± 21.6	374.4± 29.9
Week 2	410.2± 31.3	406.2± 30.9	413.8± 29.9	409.5± 31.3	410.8± 29.3	409.4± 36.9	417.6± 38.5
Week 3	442.8± 34.3	444.6± 28.7	448.7± 37.0	440.7± 31.6	453.7± 27.3	437.4± 23.0	449.6± 38.4
Week 4	455.0± 50.0	473.1± 25.9	477.7± 44.1	472.4± 34.5	488.3± 32.2	468.3± 24.1	485.1± 47.4
Week 5	489.5± 37.5	500.9± 29.5	499.0± 51.7	511.8± 33.5	522.7± 38.0	487.0± 27.8	503.7± 51.9
Week 6	511.9± 35.6	520.6± 28.9	514.2± 54.2	517.1± 38.4	545.3± 41.7	509.8± 23.5	523.2± 47.7
Week 7	520.5± 38.1	533.6± 30.7	522.9± 66.2	515.9± 60.4	563.3± 43.1	525.5± 30.2	541.8± 43.7

Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water)

includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. All values are expressed as Mean± SD. Two-way ANOVA test is performed with body weight being the dependent variable and both the interventional group and time being the factors. Significance is found with P-value<0.05.

Figure 9: Evolution of mean of body weight in grams of the seven groups at baseline and over the seven weeks interventional period



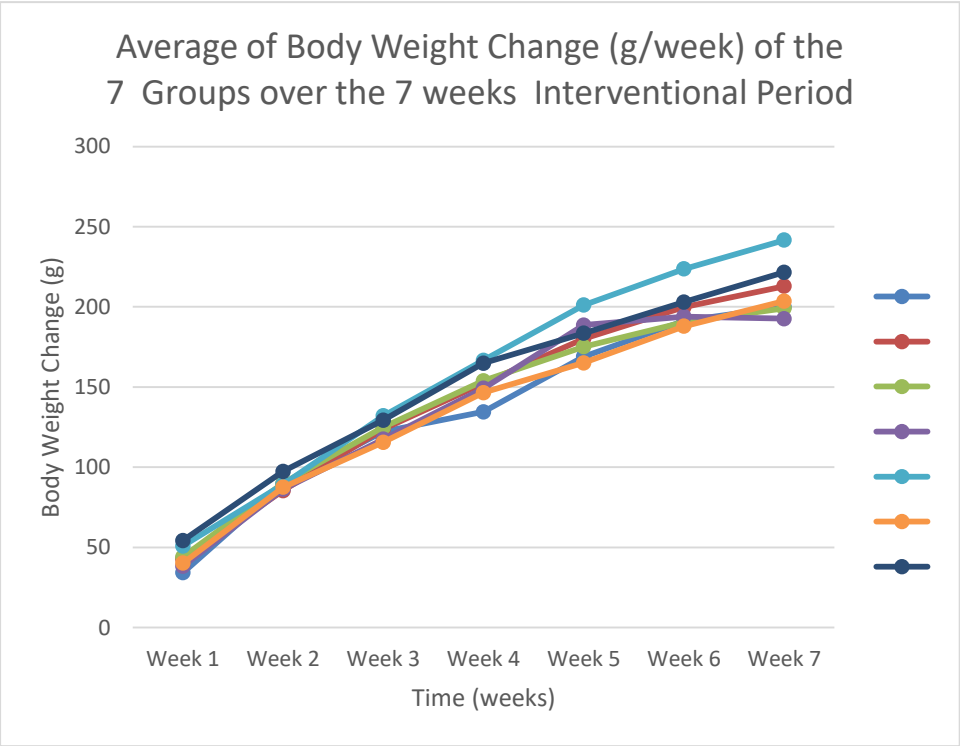
Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water.

Table 7: Mean of body weight change in grams per week for the seven groups over the 7 weeks interventional period

	Group 1 (Control) n=6	Group 2 (Asp. Diet+ Regular Water) n=7	Group 3 (Scl. Diet+ Regular Water) n=7	Group 4 (Regular Starch Diet+ Asp. Water) n=7	Group 5 (Asp. Diet+ Asp. Water) n=7	Group 6 (Scl. Water+ Regular Starch Diet) n=7	Group 7 (Scl. Diet+ Scl. Water) n=7
Week 1	34.2± 17.9	42.8± 18.4	44.1± 21.9	38.4± 12.4	50.9± 7.3	40.2± 10.5	54.3± 11.4
Week 2	89.7± 27.5	85.4± 23.3	90.0± 22.6	86.3± 16.2	89.2± 9.9	87.5± 24.6	97.4± 19.5
Week 3	122.3± 31.0	123.9± 21.9	124.9± 26.8	117.5± 16.3	132.1± 16.5	115.5± 15.4	129.4± 18.8
Week 4	134.5± 54.1	152.4± 24.8	153.9± 34.7	149.2± 15.9	166.7± 14.8	146.5± 19.6	164.9± 24.8
Week 5	169.0± 37.7	180.2± 25.7	175.2± 41.6	188.6± 36.5	201.1± 17.6	165.1± 22.2	183.5± 33.7
Week 6	191.4± 34.4	199.9± 28.4	190.4± 46.8	193.9± 20.7	223.6± 19.7	188.0± 22.9	203.0± 25.1
Week 7	200.0± 29.8	212.9± 31.9	199.2± 59.2	192.7± 56.5	241.7± 23.6	203.7± 28.8	221.6± 20.9

Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. All values are expressed as Mean± SD. Two-way ANOVA test is performed with body weight change being the dependent variable and both the interventional group and time being the factors. Significance is found with P-value<0.05.

Figure 10: Evolution of the mean of weekly body weight change (g/week) for the seven groups over the 7 weeks interventional period



Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water.

C. Body Composition

1. Body Fat

All values are expressed as Mean ± SD. Significance is found with P-value<0.05. The body fat was assessed once per week for 7 interventional weeks. The data of body fat is presented as average of weekly percentage body fat (%/ week) among the 7 groups over the 7 weeks' interventional period (table 8 and figure 11). A two-way ANOVA test is performed, % body fat is the dependent variable, and both the interventional group and time are the factors. The highest mean of % body fat is among Group 5 (Asp. Diet+ Asp. Water), and the lowest means belong to Group 1(Control Group) and Group 6 (Regular Starch Diet+ Scl. Water).

The outcome reveals that the % body fat among Group 1(Control Group) is statistically significant lower than Group 2 (Asp. Diet+ Regular Water) *(P=0.0001),* Group 3 (Scl. Diet+ Regular Water) *(P=0.034),* Group 4 (Regular Starch Diet+ Asp. Water) *(P=0.002),* Group 5 (Asp. Diet+ Asp. Water) *(P<0.0001),* and Group 7 (Scl. Diet+ Scl. Water) *(P=0.002).* Also, Group 6 (Regular Starch Diet+ Scl. Water) has a statistically significant lower %body fat than Group 2 (Asp. Diet+ Regular Water) *(P< 0.0001),* Group 3 (Scl. Diet+ Regular Water) *(P=0.016),* Group 4 (Regular Starch Diet+ Asp. Water) *(P=0.001),* Group 5 (Asp. Diet+ Asp. Water) *(P<0.0001),* and Group 7 (Scl. Diet+ Scl. Water) *(P=0.001).* In addition, %body fat of Group 5 (Asp. Diet+ Asp. Water) is statistically significant higher than Group 3 (Scl. Diet+ Regular Water) *(P=0.002),* Group 4 (Regular Starch Diet+ Asp. Water) *(P=0.029),* and Group 7 (Scl. Diet+ Scl. Water) *(P=0.027).*

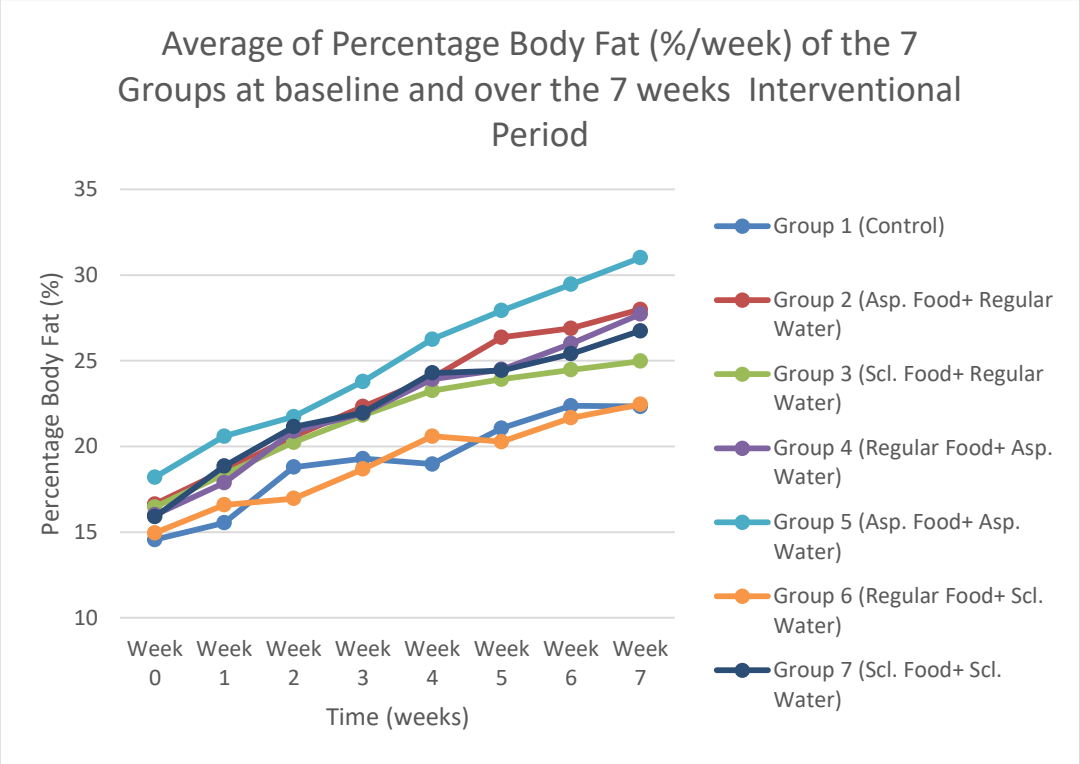
Table 8: Mean of percentage body fat in of the seven groups at baseline and over the seven weeks interventional period

	Group 1 (Control) n=6	Group 2 (Asp. Diet+ Regular Water) n=7	Group 3 (Scl. Diet+ Regular Water) n=7	Group 4 (Regular Starch Diet+ Asp. Water) n=7	Group 5 (Asp. Diet+ Asp. Water) n=7	Group 6 (Scl. Water+ Regular Starch Diet) n=7	Group 7 (Scl. Diet+ Scl. Water) n=7
Baseline	14.6± 1.5	16.6± 2.7	16.5± 2.5	16.0± 2.6	18.2± 2.0	15.0± 2.3	15.9± 2.4
Week 1	15.5± 2.4	18.6± 2.7	18.4± 3.3	17.9± 3.3	20.6± 3.2	16.6± 3.1	18.8± 1.8
Week 2	18.8± 3.4	20.6± 2.8	20.2± 3.3	20.9± 3.1	21.7± 3.8	17.0± 2.2	21.1± 2.5
Week 3	19.3± 3.8	22.3± 3.2	21.8± 3.8	21.9± 2.8	23.8± 4.8	18.7± 3.1	22.0± 3.1
Week 4	19.0± 6.2	24.0± 4.8	23.3± 4.8	23.9± 3.6	26.3± 5.6	20.6± 3.9	24.3± 3.9
Week 5	21.1± 4.6	26.4± 4.7	23.9± 6.2	24.5± 4.2	27.9± 5.9	20.3± 4.3	24.4± 4.3
Week 6	22.4± 4.3	26.9± 5.2	24.5± 6.9	26.0± 4.3	29.5± 6.3	21.7± 4.4	25.4± 4.4
Week 7	22.3± 4.9	28.0± 5.5	25.0± 8.5	27.7± 4.3	31.0± 6.8	22.5± 4.7	26.7± 3.6

Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water)

includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. All values are expressed as Mean± SD. Two-way ANOVA test is performed with percentage body fat being the dependent variable and both the interventional group and time being the factors. Significance is found with P-value<0.05.

Figure 11: Evolution of mean of percentage body fat of the seven groups at baseline and for seven interventional weeks



Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose-sweetened starch and 0.08g/500g sucralose-sweetened water.

2. Lean Body Mass

All values are expressed as Mean ± SD. Significance is found with P-value < 0.05. The lean body mass was assessed once per week for 7 interventional weeks. The data of lean body mass is presented as average of weekly percentage lean body mass (%/ week) among the 7 groups over the 7 weeks' interventional period (table 9 and figure 12). A two-way ANOVA test is performed, % lean body mass is the dependent variable, and both the interventional group and time are the factors. The highest means of % lean body mass is among Group 1 (Control) and Group 6 (Scl. Water+ Regular Starch Diet), and the lowest mean is among Group 5 (Asp. Diet+ Asp. Water).

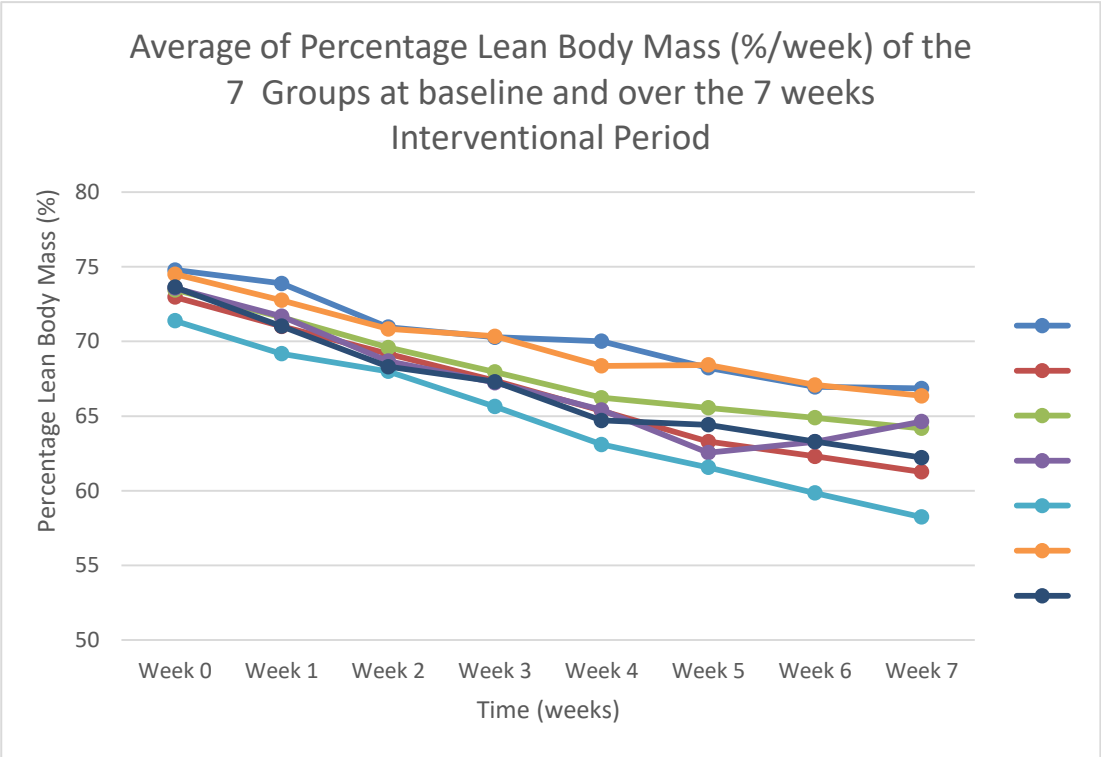
The results show that Group 1(Control Group) has a statistically significant percentage lean mass higher than Group 2 (Asp. Diet+ Regular Water) *(P=0.0003)*, Group 4 (Regular Starch Diet+ Asp. Water) *(P=0.004)*, Group 5 (Asp. Diet+ Asp. Water) *(P<0.0001)*, and Group 7 (Scl. Diet+ Scl. Water) *(P=0.001)*. Also, Group 6 (Scl. Water+ Regular Starch Diet) has a statistically significant higher mean of percentage lean body mass than Group 2 (Asp. Diet+ Regular Water) *(P=0.01)*, Group 4 (Regular Starch Diet+ Asp. Water) *(P=0.015)*, Group 5 (Asp. Diet+ Asp. Water) *(P<0.0001)*, and Group 7 (Scl. Diet+ Scl. Water) *(P=0.005)*. Additionally, regarding Group 5 (Asp. Diet+ Asp. Water), the percentage of lean body is statistically significant less than Group 3 (Scl. Diet+ Regular Water) *(P=0.001)*, Group 4 (Regular Starch Diet+ Asp. Water) *(P=0.032)*.

Table 9: Mean of percentage lean body mass of the seven groups at baseline and over the seven weeks interventional period

	Group 1 (Control) n=6	Group 2 (Asp. Diet+ Regular Water) n=7	Group 3 (Scl. Diet+ Regular Water) n=7	Group 4 (Regular Starch Diet+ Asp. Water) n=7	Group 5 (Asp. Diet+ Asp. Water) n=7	Group 6 (Scl. Water+ Regular Starch Diet) n=7	Group 7 (Scl. Diet+ Scl. Water) n=7
Baseline	74.8± 1.2	73.0± 2.4	73.5± 2.5	73.6± 2.2	71.4± 2.0	74.5± 2.3	73.6± 2.1
Week 1	73.9± 2.2	71.0± 2.3	71.6± 3.2	71.7± 3.0	69.2± 3.1	72.8± 3.1	71.0± 2.0
Week 2	71.0± 3.1	69.2± 2.7	69.6± 3.2	68.7± 2.9	68.0± 3.6	70.8± 5.0	68.3± 2.5
Week 3	70.3± 3.6	67.4± 3.2	68.0± 3.9	67.2± 2.6	65.6± 4.6	70.3± 3.0	67.3± 3.1
Week 4	70.0± 5.5	65.4± 4.4	66.2± 4.8	65.4± 3.2	63.1± 5.4	68.4± 3.9	64.7± 3.9
Week 5	68.2± 4.3	63.3± 4.5	65.6± 6.1	62.6± 6.0	61.6± 5.6	68.4± 4.1	64.4± 4.1
Week 6	67.0± 4.0	62.3± 4.9	64.9± 6.6	63.3±4.0	59.9± 5.9	67.1± 4.5	63.3± 4.5
Week 7	66.9± 4.6	61.3±5.1	64.2± 8.1	64.6± 8.9	58.2± 6.4	66.4± 4.8	62.2± 3.6

Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. All values are expressed as Mean± SD. Two-way ANOVA test is performed with percentage lean body mass being the dependent variable and both the interventional group and time being the factors. Significance is found with P-value<0.05.

Figure 12: Mean of percentage lean body mass of the seven groups at baseline and for seven interventional weeks



Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water.

D. Organs Weight

1. Heart weight

The heart weight was assessed as percentage heart weight. A one-way ANOVA test is performed to assess the association between the heart weight and the consumption of sucralose or aspartame. % heart weight is the dependent variable, and the interventional group is the factor. The outcome revealed that Group 6 (Regular Starch Diet+ Scl. Water) has the highest mean of % heart weight, and Group 5 (Asp. Diet+ Asp. Water) has the lowest mean (table 10 and figure 13). Nevertheless, there isn't a significant difference of % heart weight between all interventional groups.

2. Liver Weight

The liver weight was assessed as percentage liver weight. A one-way ANOVA test is performed to assess the association between the liver weight and the consumption of sucralose or aspartame. % liver weight is the dependent variable, and the interventional group is the factor. The highest mean of % liver weight is among Group 7 (Scl. Diet+ Scl. Water), and the lowest mean is among Group 4 (Regular Starch Diet+ Asp. Water) (table 10 and figure 14). However, the data indicates that there isn't a significant difference between the % liver weight of all interventional groups.

3. Kidney Weight

The kidney weight was assessed as percentage kidney weight. A one-way ANOVA test is performed to assess the association between the kidney weight and the

consumption of sucralose or aspartame. % kidney weight is the dependent variable, and the interventional group is the factor. The highest mean of % kidney

weight is among Group 5 (Asp. Diet+ Asp. Water), and the lowest is among Group 6 (Regular Starch Diet+ Scl. Water) (table 10 and figure 15). Mean percentage kidney weight in Group 6 (Regular Starch Diet+ Scl. Water) is close to significant higher than Group 5 (Asp. Diet+ Asp. Water) *(P=0.055).* In spite of that, the difference of mean % kidney weight is not statistically significant different between all the groups.

4. Epididymal Adipose Tissue Weight

The epididymal adipose tissue weight was assessed in percentage epididymal adipose tissue weight. A one-way ANOVA test is performed to assess the association between the epididymal adipose tissue weight and the consumption of sucralose or aspartame sweeteners. % Epididymal adipose tissue weight is the dependent variable, and the interventional group is the factor. The results show that the highest mean of %epididymal adipose tissue weight is found in Group 5 (Asp. Diet+ Asp. Water), and the lowest mean is found in Group 1 (Control) (table 10 and figure 16). Group 5 (Asp. Diet+ Asp. Water) has a statistically significant higher mean of % epididymal adipose tissue weight than Group 1 (Control) *(P=0.027),* and Group 6 (Scl. Water+ Regular Starch Diet) (*P=0.017).*

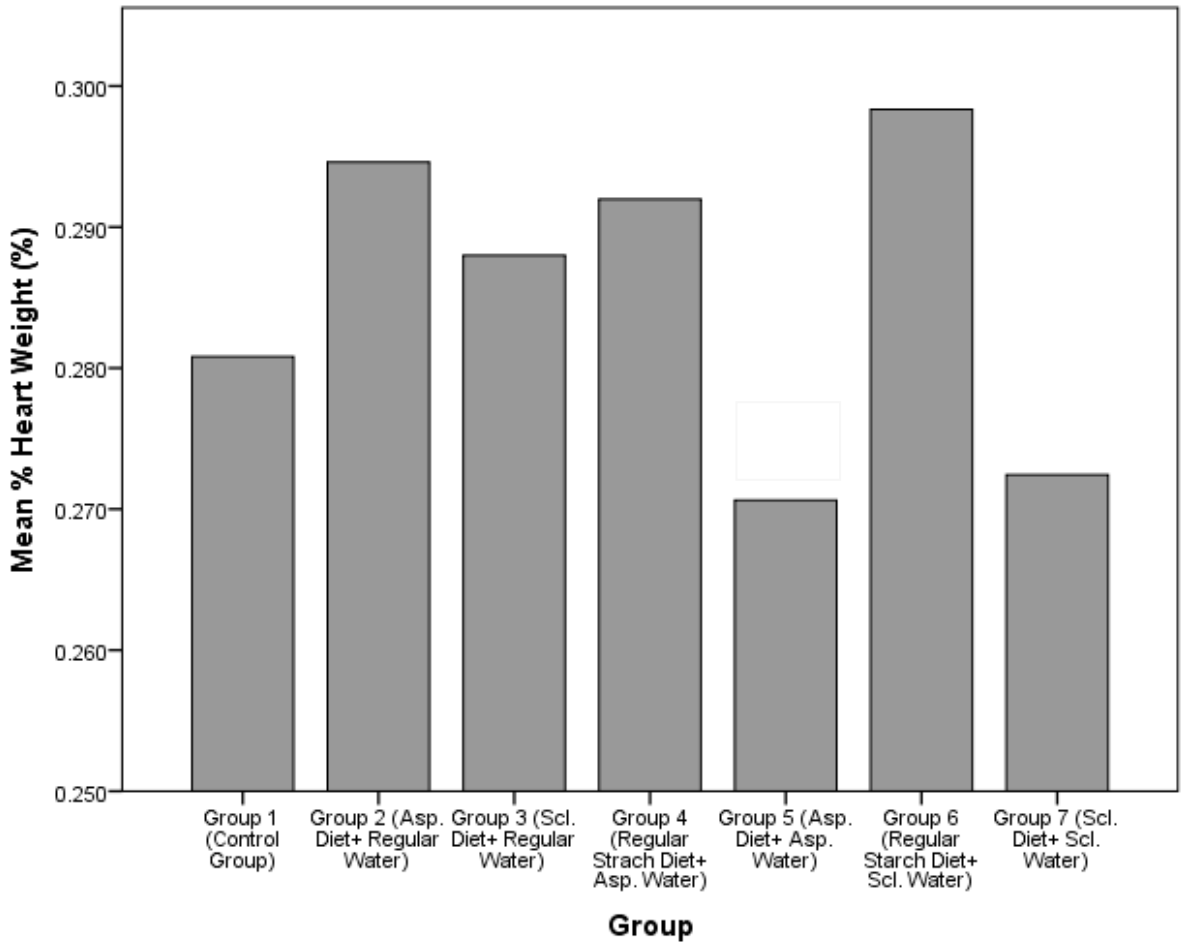
Table 10: Average percentages of heart, liver, kidney, and epididymal adipose tissue weights of the seven groups

	Group 1 (Control) n=6	Group 2 (Asp. Diet+ Regular Water) n=7	Group 3 (Scl. Diet+ Regular Water) n=7	Group 4 (Regular Starch Diet+ Asp. Water) n=7	Group 5 (Asp. Diet+ Asp. Water) n=7	Group 6 (Scl. Water+ Regular Starch Diet) n=7	Group 7 (Scl. Diet+ Scl. Water) n=7	Significance of mean difference between groups (P-value)
Average Percentage Heart Weight (%)	0.281±0.028	0.295±0.035	0.288±0.019	0.292±0.022	0.271±0.014	0.298±0.019	0.272±0.019	*P=0.190*
Average Percentage Liver Weight (%)	2.61± 0.26	2.73± 0.25	2.78± 0.30	2.59± 0.30	2.78± 0 .25	2.65± 0.28	2.82± 0.45	*P= 0.727*
Average Percentage Kidney Weight (%)	0.62± 0.02	0.59± 0.03	0.61± 0.04	0.62± 0.03	0.57± 0.03	0.64± 0.07	0.60± 0.04	*P= 0.074*
Average Percentage Epididymal Adipose Tissue Weight (%)	1.71± 0.33 [a]	2.19± 0.35	2.18± 0.71	2.20± 0.30	2.20± 0.55 [a b]	2.60± 0.30 [b]	1.79± 0.47	*P= 0.019*

a b statistically significant difference exists between the groups

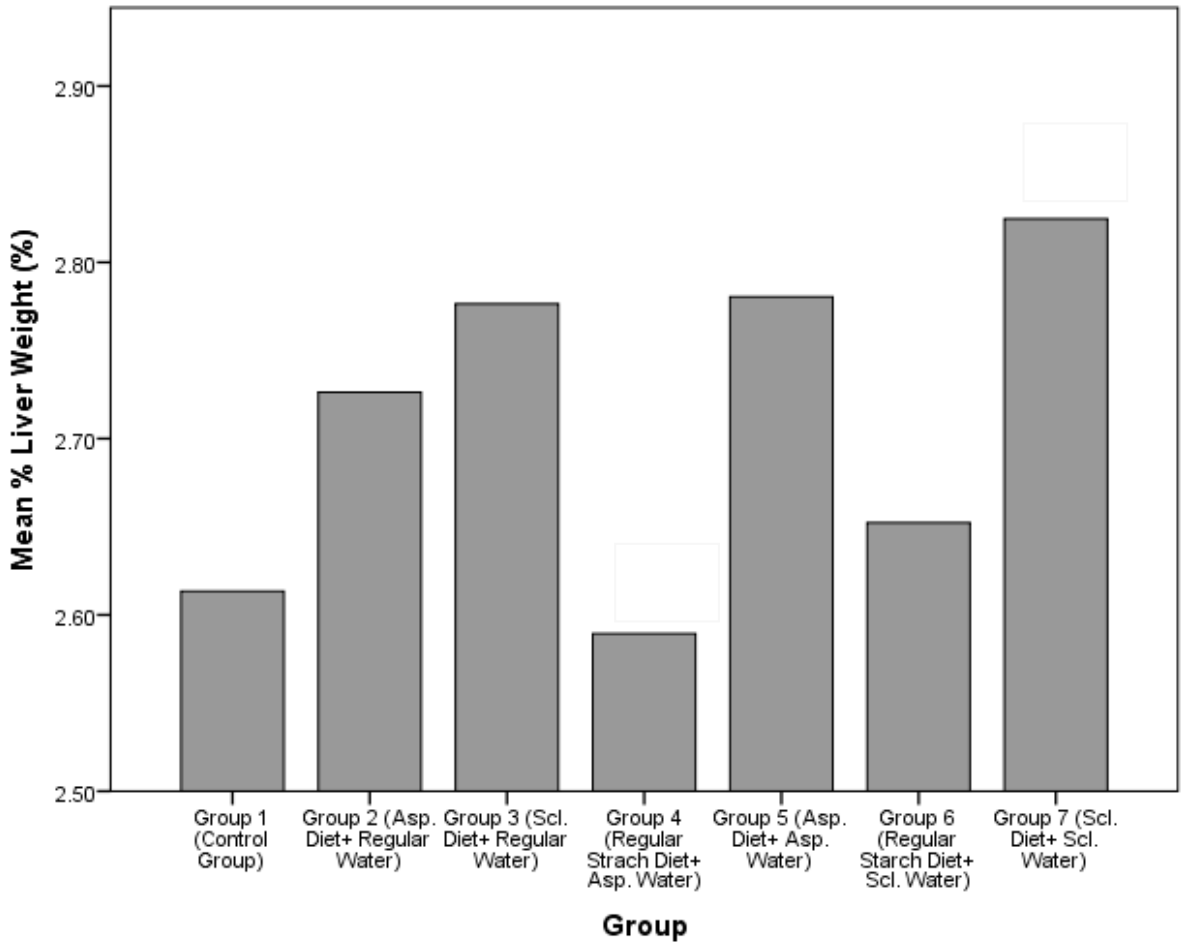
Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. All values are expressed as Mean± SD. A One-Way ANOVA test was performed. Significance is found at P-value<0.05.

Figure 13: Average percentage heart weight of the seven groups



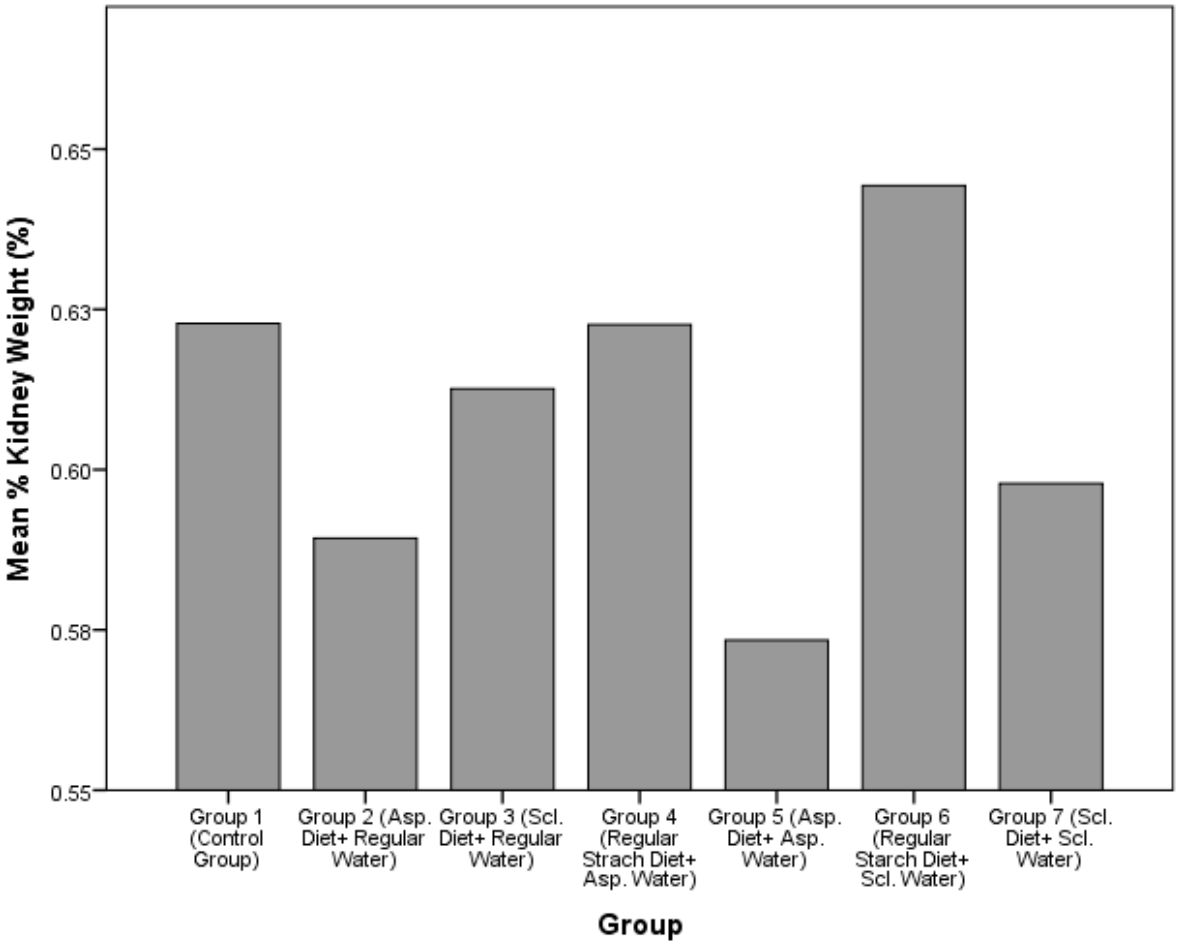
Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. A one- way ANOVA test was performed.

Figure 14: Mean percentage liver weight of the seven groups



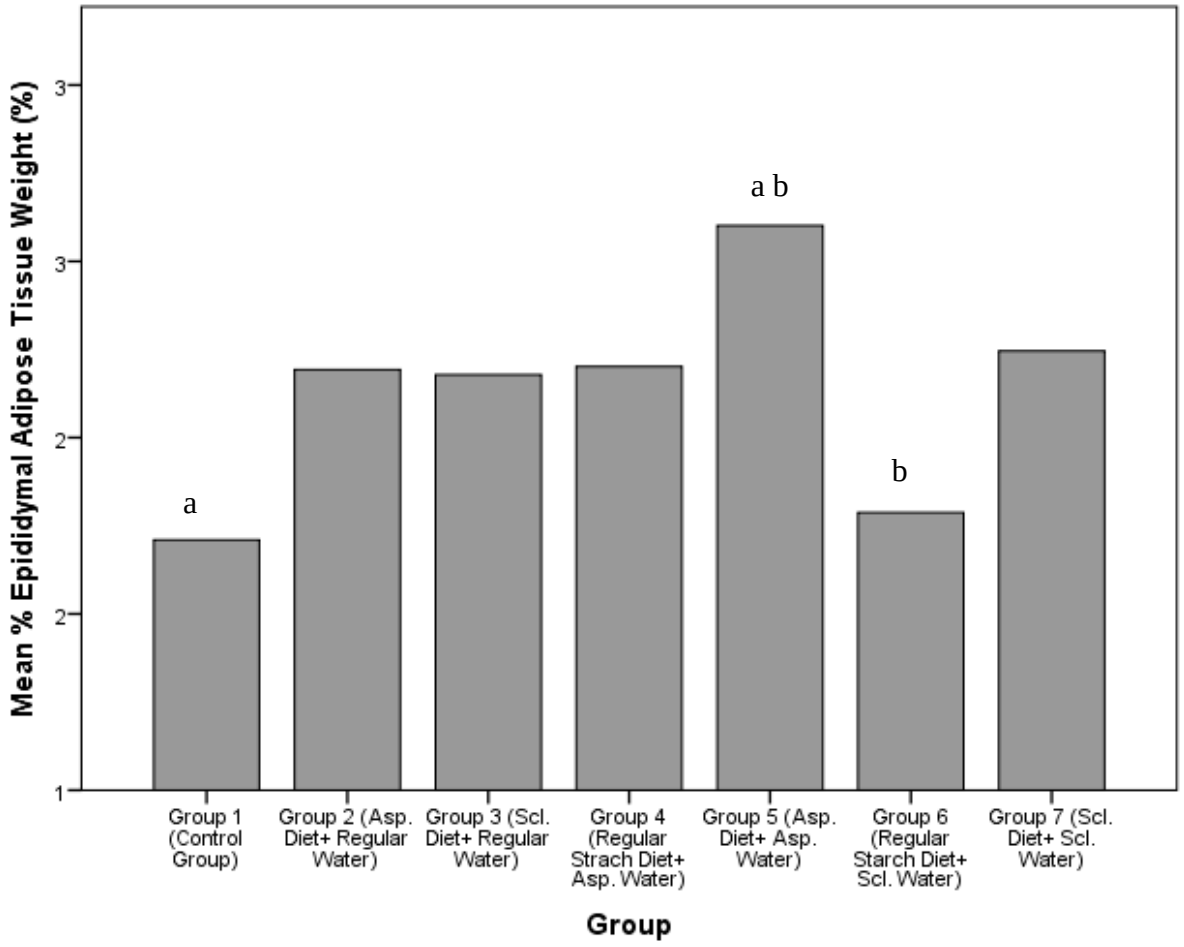
Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. A one- way ANOVA test was performed.

Figure 15: Average percentage kidney weight of the seven groups



Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. A one- way ANOVA test was performed.

Figure 16: Average percentage epididymal adipose tissue weight



a b statistically significant difference exists between the groups
Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. A one- way ANOVA test was performed. Significance is found at P-value < 0.05.

E. Plasma Analysis

1. Fasting Insulin

All values are expressed as Mean ± SD. A one-way ANOVA test is performed to assess the association between insulin and sucralose/ aspartame. Fasting insulin is the dependent variable and the interventional group was the factor. The highest mean of fasting insulin was found among Group 2 (Asp. Diet+ Regular Water) and Group 5 (Asp. Diet+ Asp. Water). The lowest mean of fasting insulin is found among Group 1(Control Group) and Group 4 (Regular Starch Diet+ Asp. Water) (table 11 and figure 17). However, the mean difference of fasting insulin was not statistically significant.

2. Metabolic Markers

a. Fasting Glucose

A one-way ANOVA test is performed to assess the association between fasting blood glucose and sucralose/ aspartame. Fasting blood glucose is the dependent variable, and the interventional group is the factor. The results show that the highest mean of fasting blood glucose is among Group 5 (Asp. Diet+ Asp. Water), and the lowest mean is among Group 1 (Control) (table 12 and figure 18). Nevertheless, the mean difference between the groups is not significant.

b. Triglyceride

A one-way ANOVA test is performed to assess the association between plasma triglyceride and sucralose or aspartame. The results show that the highest mean of triglyceride is found among Group 5 (Asp. Diet+ Asp. Water), and the lowest mean is among Group 1 (Control) (table 13 and figure 19). In spite of that, the difference of mean triglyceride was not significant between the groups.

c. Total Cholesterol

A one-way ANOVA test is performed to assess the association between total serum cholesterol and artificial sweeteners. The data indicates that the highest mean of total cholesterol is among Group 3 (Scl. Diet+ Regular Water) and Group 5 (Asp. Diet+ Asp. Water), and the lowest mean is among Group 7 (Scl. Diet+ Scl. Water) (table 13 and figure 20). The mean difference of total cholesterol between the groups was not statistically significant*(P=0.067).*

d. HDL

A one-way ANOVA test is performed to assess the association between HDL and sucralose or aspartame. The results indicate that Group 3 (Scl. Diet+ Regular Water) has the highest mean of HDL. Whereas Group 6 (Regular Starch Diet+ Scl. Water) and Group 7 (Scl. Diet+ Scl. Water) have the lowest mean (Table 13 and figure 21). There isn't a statistically significant difference between the groups.

e. LDL

The Friedewald equation was used to estimate LDL:

LDL= high density lipoprotein- total cholesterol- (triglyceride/ 5)

A one-way ANOVA test is performed to assess the association between LDL and artificial sweeteners. The data shows that the highest mean of LDL is among Group 6 (Regular Starch Diet+ Scl. Water), and the lowest mean is among Group 2 (Asp. Diet+ Regular Water) (table 13 and figure 22). There isn't a significant difference between the groups.

f. VLDL

VLDL was calculated by dividing triglyceride by five.

A one-way ANOVA test is performed to assess the association between VLDL and sucralose or aspartame. The data shows that the highest mean of VLDL is among the Group 5 (Asp. Diet+ Asp. Water), and the lowest is among Group 6 (Regular Starch Diet+ Scl. Water) (table 13 and figure 23). However, the mean difference of VLDL is not significant between the groups.

g. Albumin

A one-way ANOVA test is performed to assess the association between albumin and artificial sweeteners. The highest mean of albumin is among Group 4 (Regular Starch Diet+ Asp. Water) and Group 6 (Regular Starch Diet+ Scl. Water). The lowest mean is among Group 3 (Scl. Diet+ Regular Water) (table 14). However, the mean of albumin is not statistically different between groups *(P=0.693*).

h. Creatinine

A one-way ANOVA test is performed to assess the association between creatinine and sucralose or aspartame. The mean creatinine was the highest among Group 4 (Regular Starch Diet+ Asp. Water) and Group 6 (Regular Starch Diet+ Scl. Water). It was the lowest among Group 5 (Asp. Diet+ Asp. Water) (table 14). There isn't a statistically significant difference of mean creatinine between the groups *(P=0.815)*.

i. BUN

A one-way ANOVA test is performed to assess the association between BUN and artificial sweeteners. The results show that the highest mean BUN is among Group 7 (Scl. Diet+ Scl. Water), and the lowest mean is among Group 6 (Regular Starch Diet+

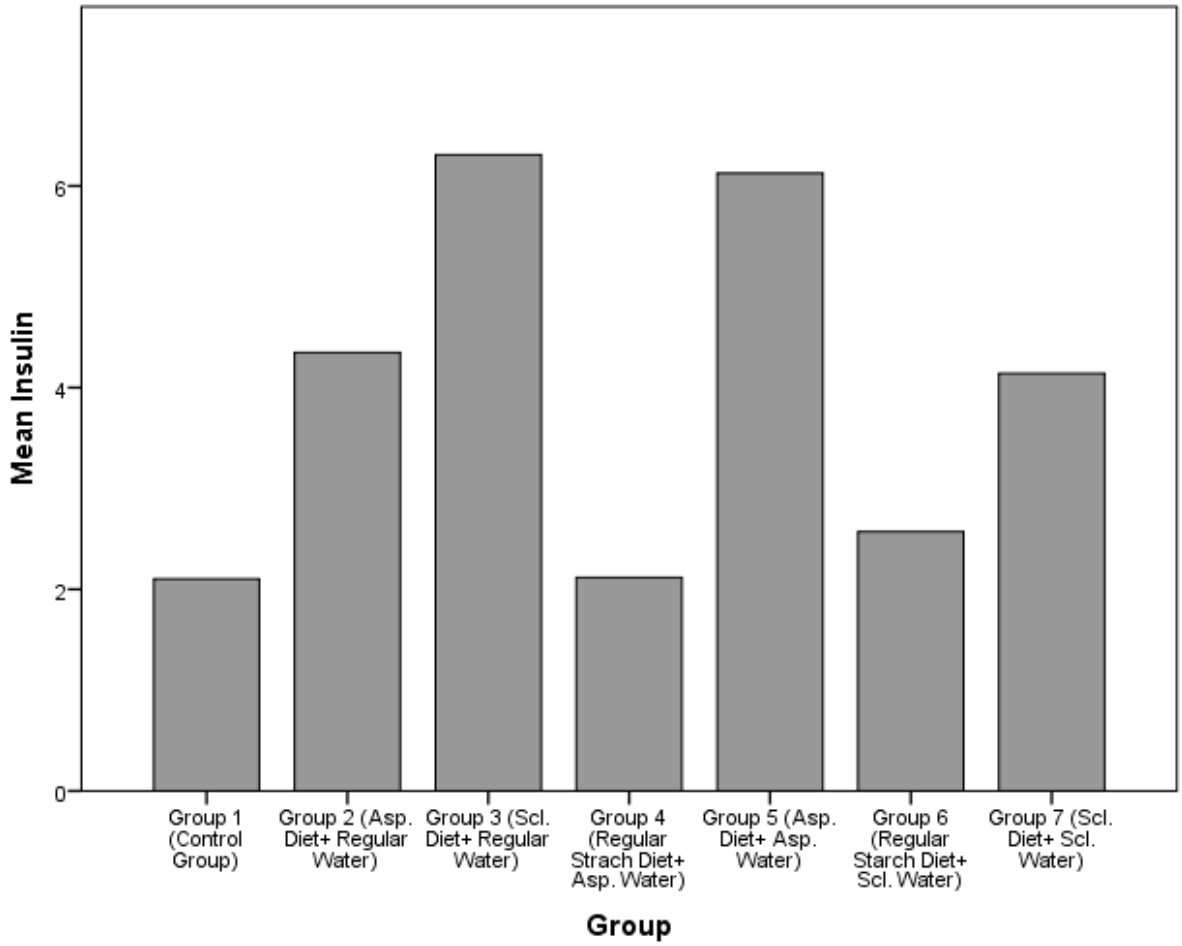
Scl. Water) (table 14). However, the difference of mean BUN is not significant between the groups.

Table 11: Mean of fasting plasma insulin in ng/ ml of the seven groups

	Group 1 (Control) n=6	Group 2 (Asp. Diet+ Regular Water) n=7	Group 3 (Scl. Diet+ Regular Water) n=7	Group 4 (Regular Starch Diet+ Asp. Water) n=7	Group 5 (Asp. Diet+ Asp. Water) n=7	Group 6 (Scl. Water+ Regular Starch Diet) n=7	Group 7 (Scl. Diet+ Scl. Water) n=7	Significance of mean difference between groups (P-value)
Average Fasting Insulin (ng/ ml)	2.10± 3.64	4.35± 3.20	6.31± 6.61	2.12± 3.23	6.13± 3.64	2.57± 5.38	4.14± 5.02	*P= 0.413*

Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. A one- way ANOVA test was performed.

Figure 17: Mean of fasting plasma insulin in ng/ ml of the seven groups



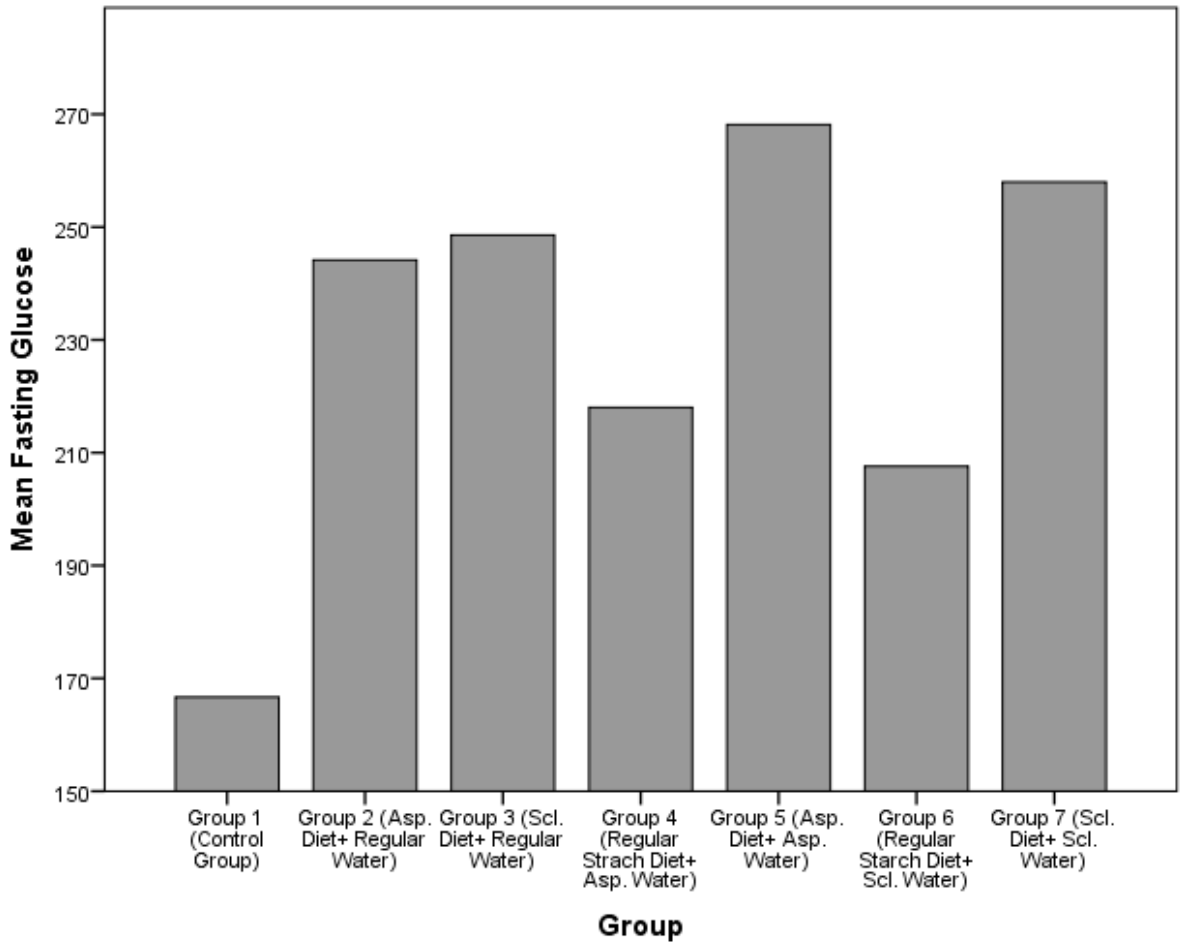
Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water.

Table 12: Mean fasting plasma glucose in mg/ dl among the seven groups

	Group 1 (Control) n=6	Group 2 (Asp. Diet+ Regular Water) n=7	Group 3 (Scl. Diet+ Regular Water) n=7	Group 4 (Regular Starch Diet+ Asp. Water) n=7	Group 5 (Asp. Diet+ Asp. Water) n=7	Group 6 (Scl. Water+ Regular Starch Diet) n=7	Group 7 (Scl. Diet+ Scl. Water) n=7	Significance of mean difference between groups (P-value)
Average Fasting Serum Glucose (mg/ dl)	166.7± 40.8	244.1± 48.4	248.6± 102.3	218.0± 47.3	268.1± 41.5	207.6± 39.3	258.0± 93.3	*P= 0.100*

Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. A one- way ANOVA test was performed.

Figure 18: Mean fasting plasma glucose in mg/ dl among the seven groups



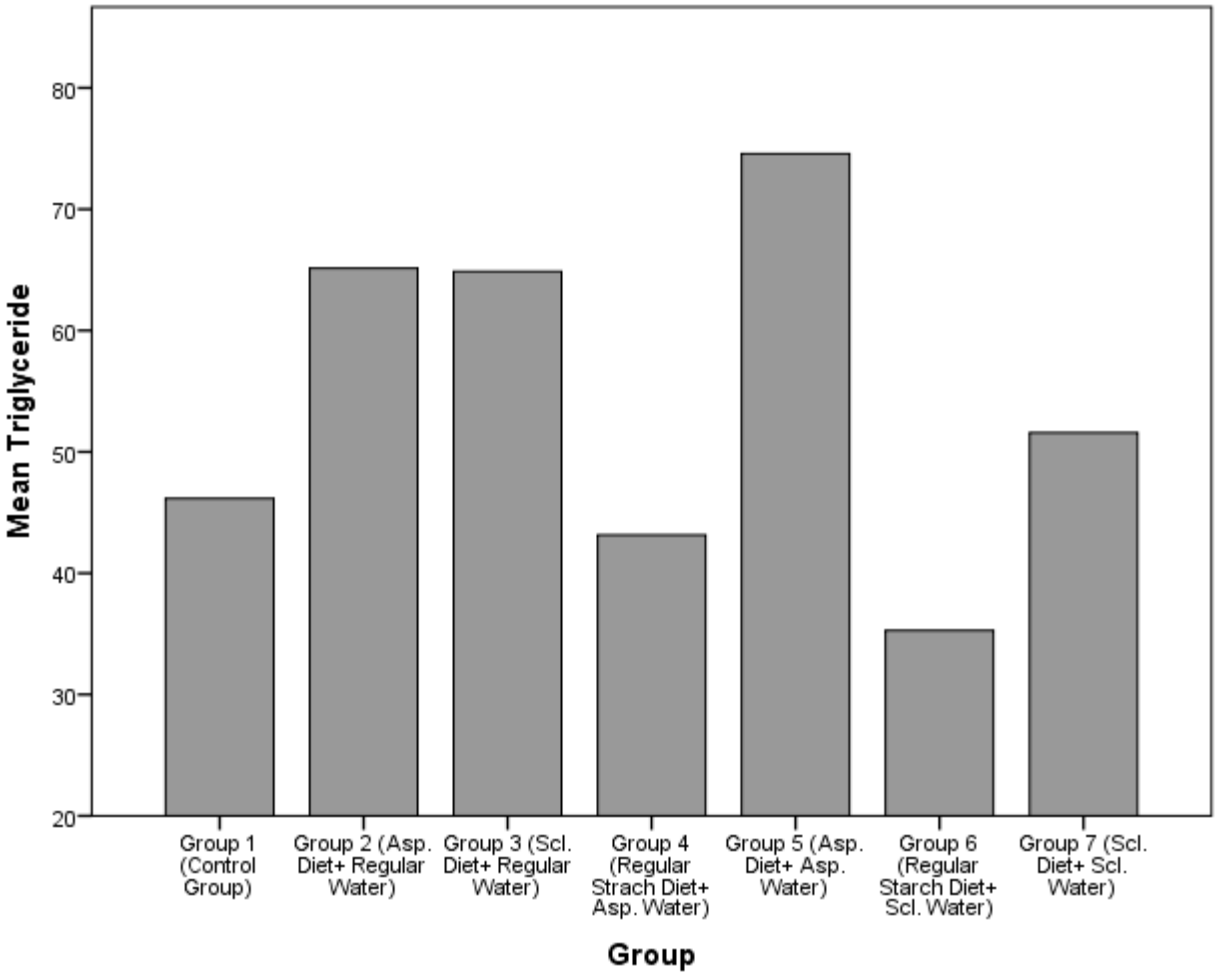
Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. A one-way ANOVA test was performed.

Table 13: Mean of lipid profile in mg/ dl among the seven groups

	Group 1 (Control) n=6	Group 2 (Asp. Diet+ Regular Water) n=7	Group 3 (Scl. Diet+ Regular Water) n=7	Group 4 (Regular Starch Diet+ Asp. Water) n=7	Group 5 (Asp. Diet+ Asp. Water) n=7	Group 6 (Scl. Water+ Regular Starch Diet) n=7	Group 7 (Scl. Diet+ Scl. Water) n=7	Significance of mean difference between groups (P-value)
Average Triglyceride	46.2± 20.8	65.1± 9.0	64.9± 22.9	43.1± 8.4	74.6± 67.6	35.3± 28.8	51.6± 21.7	*P= 0.248*
Average Total Cholesterol	64.8± 10.2	68.4± 10.6	74.0± 11.7	66.7± 9.9	74.4± 9.9	61.9± 6.9	59.7± 11.0	*P= 0.067*
Average High- Density Lipoprotein	42.8± 8.4	47.4± 8.9	50.9± 4.9	45.9± 6.6	49.6± 5.8	41.4± 4.6	41.3± 10.4	*P= 0.100*
Average Low-Density Lipoprotein	12.77± 6.63	7.97± 6.35	10.17± 7.17	12.23± 5.68	9.94± 3.97	13.37± 4.71	8.11± 4.82	*P= 0.434*
Average Very low-Density Lipoprotein	9.23± 4.34	13.03± 5.76	12.97± 13.52	8.63± 1.69	14.91± 4.57	7.06± 1.81	10.31± 4.16	*P= 0.248*

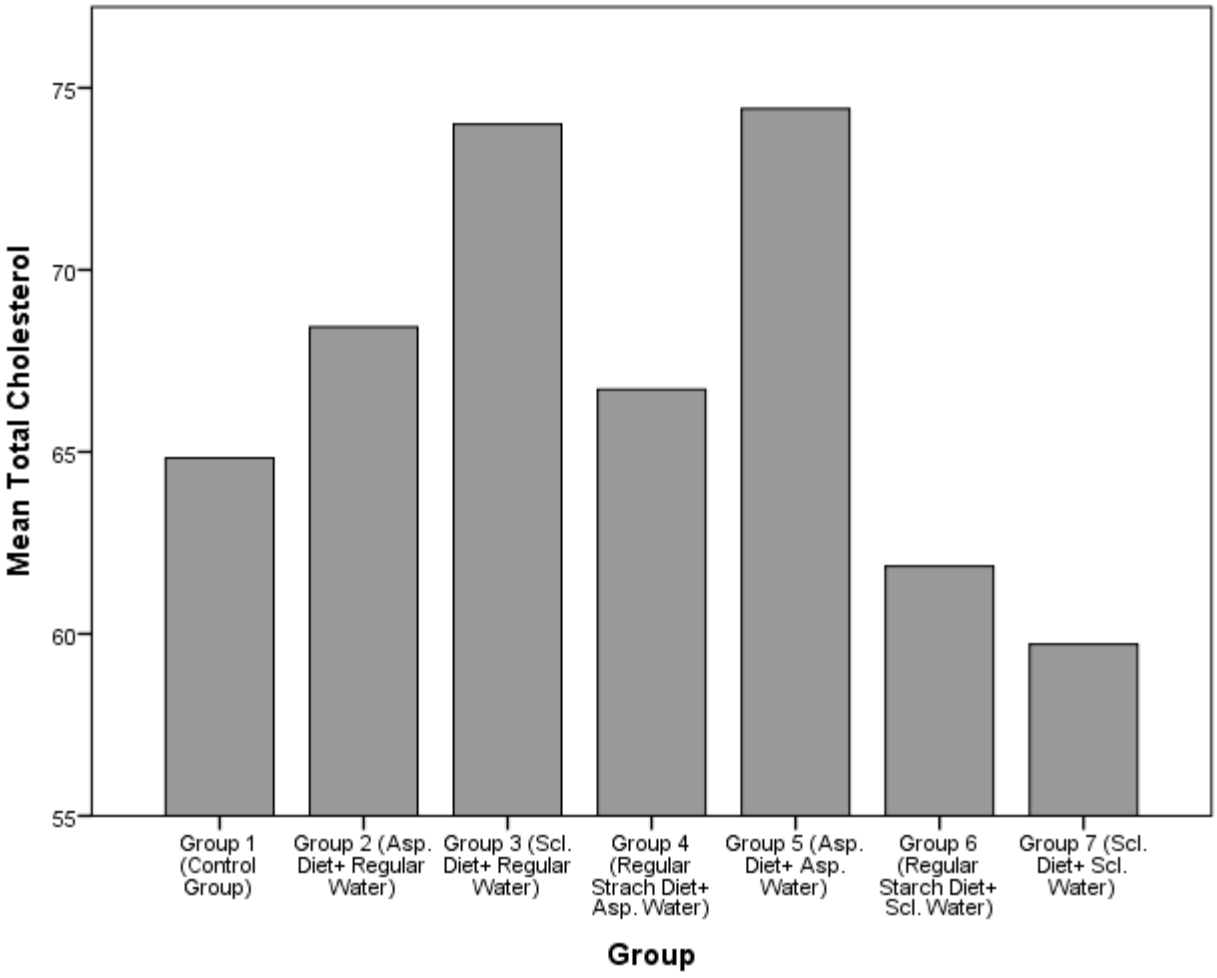
Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. A one-way ANOVA test was performed.

Figure 19: Average plasma triglyceride in mg/dl of the seven groups



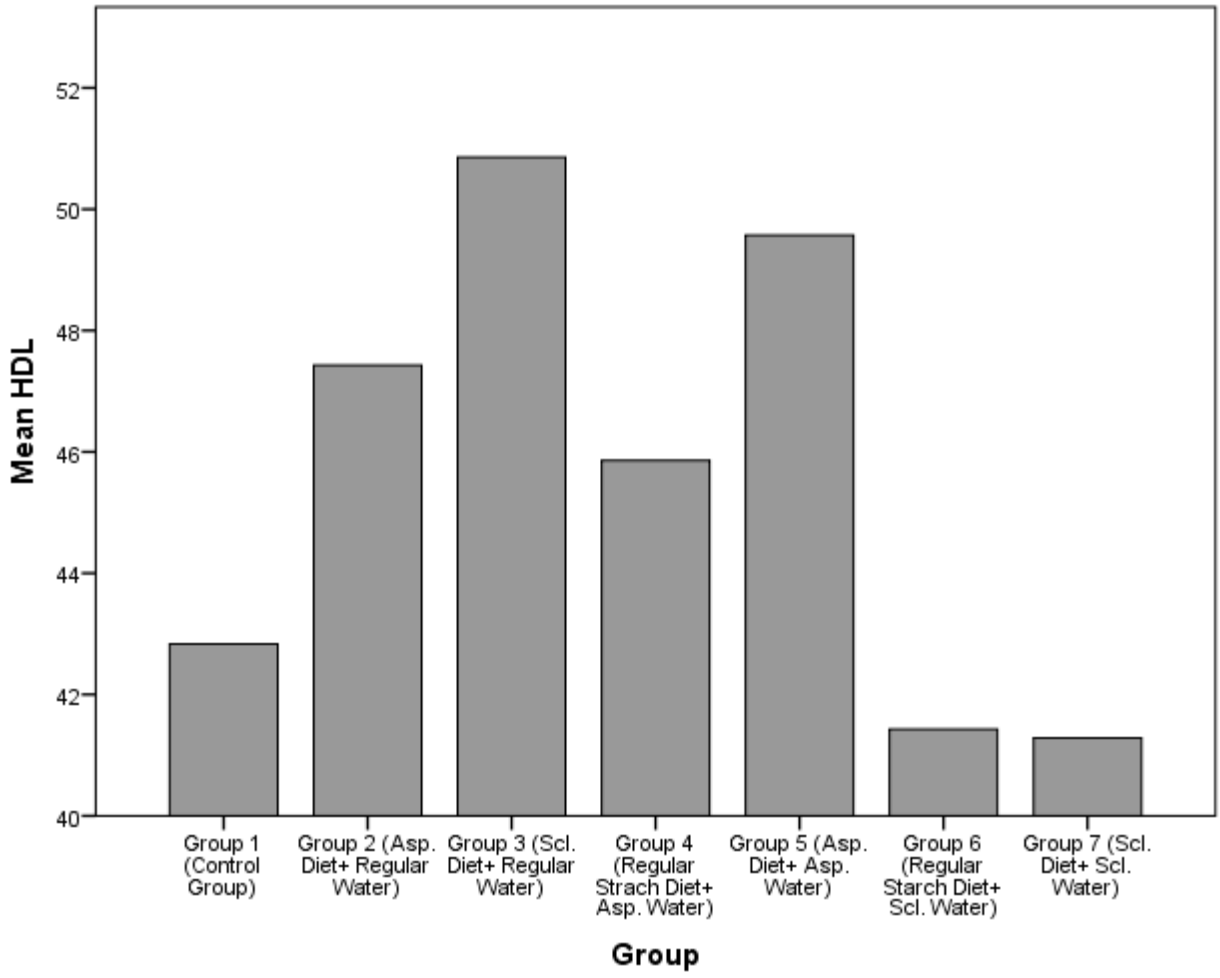
Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. A one-way ANOVA test was performed.

Figure 20: Average plasma total cholesterol in mg/ dl of the seven groups



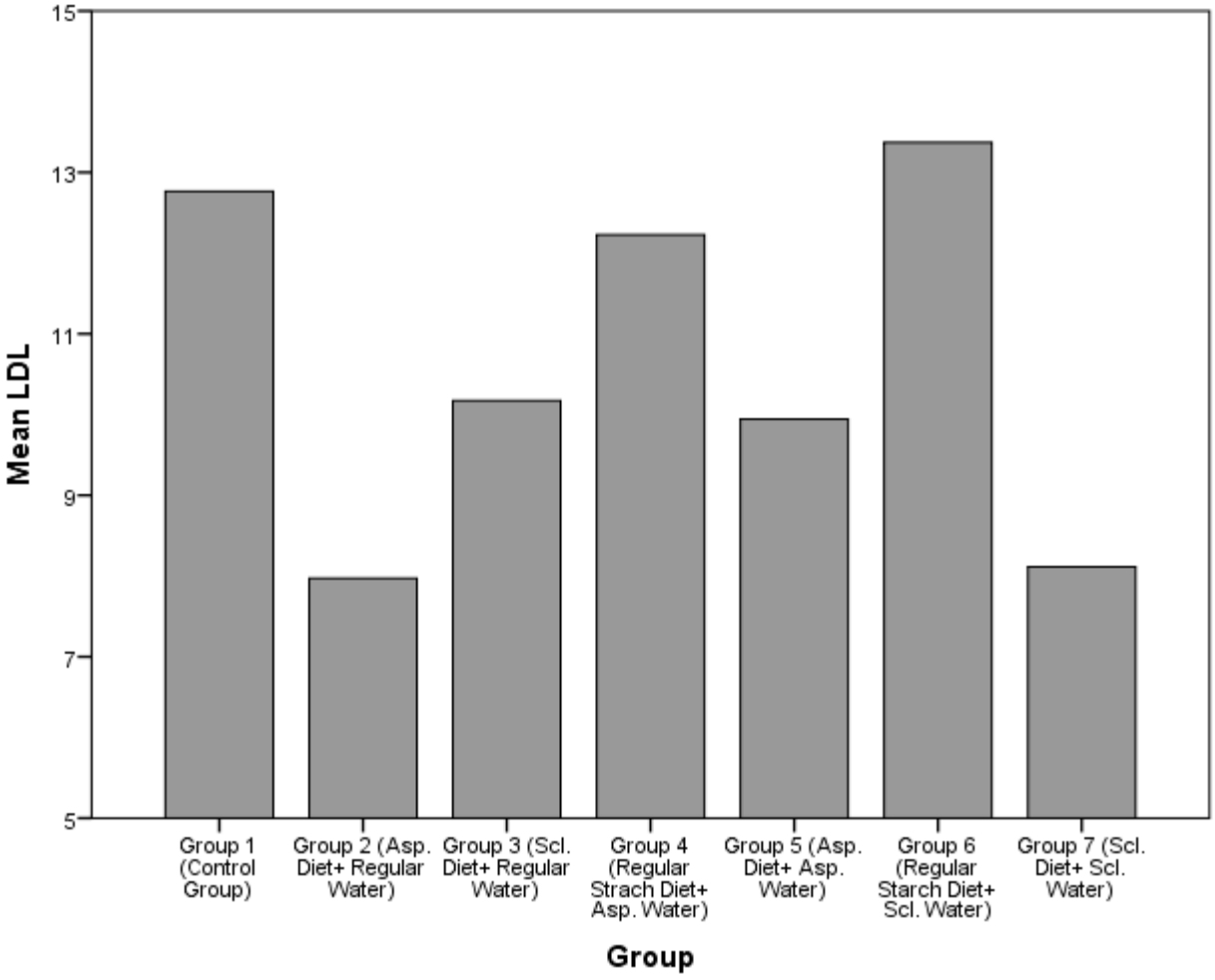
Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. A one-way ANOVA test was performed.

Figure 21: Average HDL in mg/dl of the seven groups



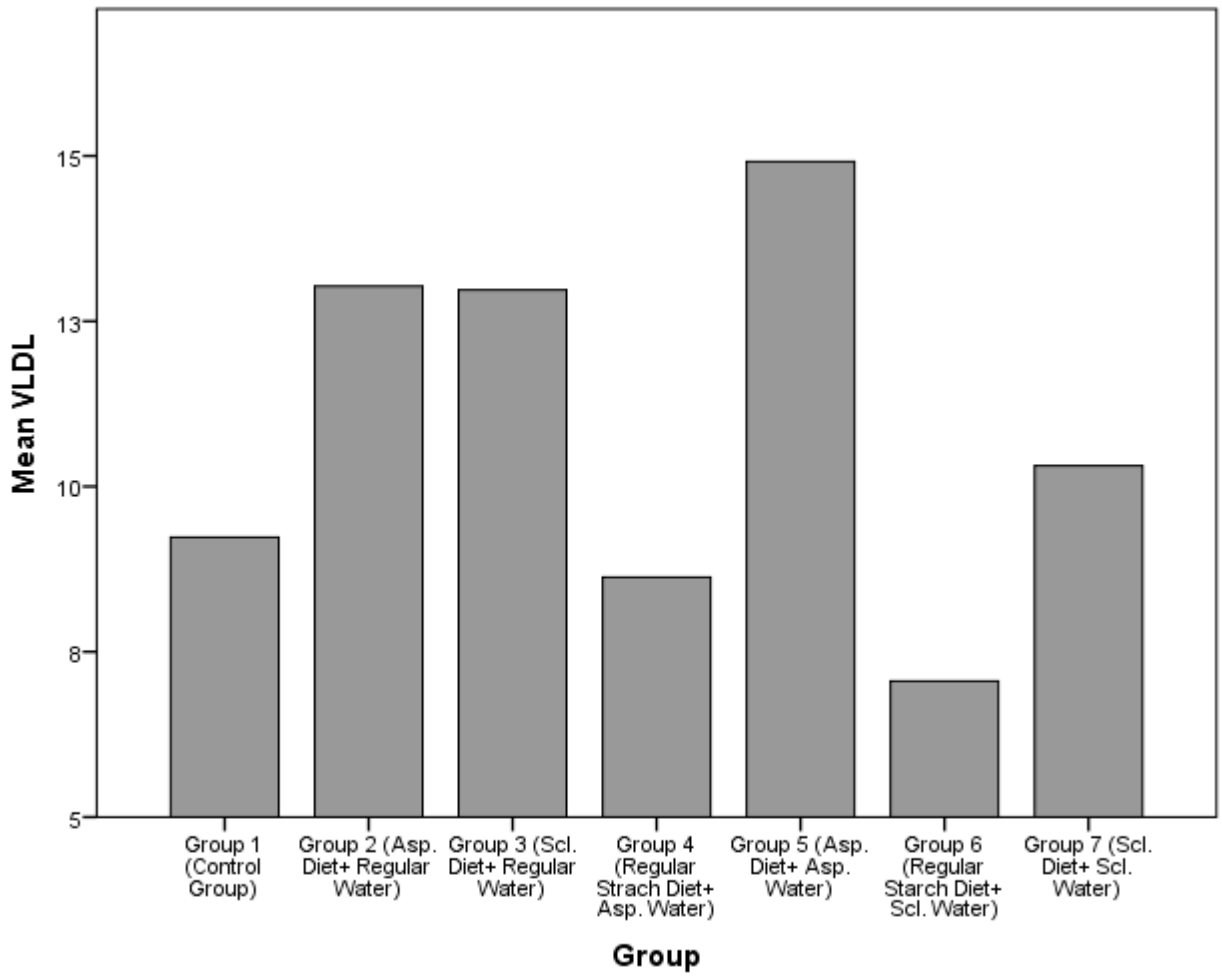
Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. A one-way ANOVA test was performed.
(HDL: High- Density Lipoprotein)

Figure 22: Average LDL in mg/dl of the seven groups



Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. A one-way ANOVA test was performed.
(LDL: Low- Density Lipoprotein)

Figure 23: Average VLDL in mg/dl of the seven groups



Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. A one-way ANOVA test was performed.
(VLDL: Very Low- Density Lipoprotein)

Table 14: Average albumin, creatinine, and blood urea nitrogen

	Group 1 (Control) n=6	Group 2 (Asp. Diet+ Regular Water) n=7	Group 3 (Scl. Diet+ Regular Water) n=7	Group 4 (Regular Starch Diet+ Asp. Water) n=7	Group 5 (Asp. Diet+ Asp. Water) n=7	Group 6 (Scl. Water+ Regular Starch Diet) n=7	Group 7 (Scl. Diet+ Scl. Water) n=7	Significance of mean difference between groups (P-value)
Average Albumin (mg/ dl)	3.38± 0.17	3.3± 0.21	3.26± 0.33	3.47± 0.21	3.46± 0.28	3.47± 0.38	3.37±0.26	*P=0.693*
Average Creatinine (mg/ dl)	0.250± 0.084	0.243± 0.053	0.243± 0.079	0.257± 0.079	0.200± 0.100	0.257± 0.079	0.229±0.049	*P=0.815*
Average Blood Urea Nitrogen (mg/ dl)	13.67± 1.37	13.71± 2.29	14.14± 1.35	14.29± 2.36	13.57± 1.99	13.43± 3.41	14.43± 1.51	P=0.967

Group 1(Control Group) includes diet with starch and regular unsweetened water; Group 2 (Asp. Diet+ Regular Water) includes diet with 0.5g/kg aspartame-sweetened starch and unsweetened regular water; Group 3 (Scl. Diet+ Regular Water) includes diet with 0.16 g/kg sucralose-sweetened starch and unsweetened regular water; Group 4 (Regular Starch Diet+ Asp. Water) contains diet with starch and 0.25g/500g aspartame-sweetened water; Group 5 (Asp. Diet+ Asp. Water) consists of diet with 0.5g/kg aspartame- sweetened starch with 0.25g/500g aspartame-sweetened water; Group 6 (Regular Starch Diet+ Scl. Water) contains diet with starch and 0.08g/500g sucralose-sweetened water; Group 7 (Scl. Diet+ Scl. Water) includes diet with 0.16g/kg sucralose- sweetened starch and 0.08g/500g sucralose-sweetened water. A one-way ANOVA test was performed.

CHAPTER V

DISCUSSION

There is continuing controversy about the safety of AS. Some epidemiological studies have found that AS is associated with obesity and metabolic diseases, other studies did not find associations. In our rodent study, the consumption of the artificial sweeteners aspartame and sucralose showed significant metabolic disturbances. The severity of the effect of AS on the metabolism depends on the type and the dose of the sweetener. The study has several strengths. The interventions and the design of the study mimic the routine life. The intervention for all the study groups was isocaloric. The dose of the sweetener used was depending on the level of sweetness of the AS compared to sucrose. Sucralose is more sweet than aspartame; therefore, the dose of sucralose was less than the dose of aspartame. The literature includes a lot of research studies conducted on artificial sweeteners, but very few had a control group, used sweeteners in both fluids and food to mimic real life situations, and used adequate and clinical significant doses. Most of the studies that revealed negative and toxic effects of AS used huge and clinically insignificant doses. Our aim is to mimic real life situations where AS are present in small doses in soft drinks and food to replace sugar.

The food intake was not statically significant different between the control (starch) group and the aspartame or sucralose groups. Which is contrary to the literature suggesting that the AS impair the cephalic response to ingested food, disrupt the food reward pathway, increase appetite, increase cravings, and increase caloric consumption(9, 33). Schiffman et al. said that the researchers are not sure yet if sucralose can traverse the blood–brain barrier to reach the hypothalamus that controls

appetite(12). Prokić et al. claimed that aspartame is associated with an increase in appetite because phenylalanine, the metabolite of aspartame increases the endogenous anorectic agent cholecystokinin (CCK). Also, aspartate, another metabolite of aspartame crosses the arcuate nucleus in the brain that synthesizes neuropeptide Y, which stimulates carbohydrate intake(34).

The consumption of aspartame was associated with a significant increase in body weight (g) and body weight change independently of food intake, compared to the consumption of starch without aspartame. Also, it was markable that when the dose of aspartame increases, the effect of aspartame on body weight, and body weight change increases. The effect of aspartame on body weight is more pronounced than the effect of sucralose; however, the difference was not significant in higher doses of sucralose. In fact, small doses of sucralose did not have significant effects on body weight and body weight change like aspartame has. The effect of sucralose on body weight was not significantly different from the effect of starch without sucralose. However, it was noticeable that when the dose of sucralose increases, the body weight and body weight change increase independently of food intake. It is hypothesized that since the dose of sucralose used in the study is less than the dose of aspartame, the sucralose did not show a pronounced effect as aspartame did in small doses. This marked increase in body weight after 8 interventional weeks is due to the increase of fat mass. Both sucralose and aspartame intake are associated with a significant alteration in body composition. Even in small doses, aspartame was associated with a significant increase in % body fat accompanied with a significant decrease in %lean mass compared to starch. In case of sucralose, even thought we did not see a significant weight change; sucralose affected the body composition significantly, and it increased the fat in the body. In higher dose

of sucralose, the significant increase in body fat was accompanied with a significant decrease in lean mass. AS are commonly thought to be healthy substitutes of sugar, especially in case of the metabolically ill individuals and the ones that enjoy the sweet taste. It is thought that replacing the caloric sugar with AS contribute in decreasing the energy intake; therefore, losing weight. However, the outcome of the study reveals that a calorie is not a calorie. Even though AS provide zero calorie since they are consumed in tiny doses, but they were associated with weight gain, increase in body fat, and decrease in lean mass. Also, the weight of epididymal fat increased in case of sucralose and aspartame intake compared to the control group. The effect was more pronounced when the dose increases; however, the difference is only significant in case of higher doses of aspartame (further details will be discussed later). Previous rodent studies may have yielded results showing that aspartame and sucralose have negative effect on weight and body composition; nevertheless, those studies used a clinical insignificant dose of aspartame and sucralose, that goes beyond the usual human intake, and they did not distinguish among the different types of AS, but they considered them as a group(16, 35). Our study included a control group, aspartame and sucralose groups where aspartame and sucralose, in clinically significant doses were placed in both water and food to mimic the trend of human consumption of AS as much as possible. Our study indicated that AS are not beneficial for weight loss and weight maintenance. Many possible explanations exist. First of all, the increase in body weight, increase in body fat, and decrease in lean mass could be due to the alteration in glucose metabolism, and the insulin sensitivity. In fact, the outcome of the study revealed that the fasting blood glucose is higher in case of aspartame or sucralose intake compared with the control group. The effect becomes more pronounced when the dose increases.

The effect of aspartame and sucralose on blood glucose and insulin was not statistically significant. Similarly, aspartame and sucralose were associated with hyperinsulinemia; nevertheless, it was not statistically significant. In spite of the insignificance of the results, it is hypothesized that sucralose and aspartame started to affect the glucose homeostasis, the insulin sensitivity, and the pancreatic ß cells function. Longer duration of the study, higher doses of AS, metabolically ill study population could have shown a statistically significant effect(36). The disruption in glucose homeostasis and the hyperinsulinemia resulted from the intervention could be related to the mechanism of sweet taste perception. Extensive research was done on the mechanism of sweet taste perception. The sweet taste, whether is coming from nutritive or non-nutritive sweetener is a stimulus. The sweet taste receptors T1R2 and T1R3 predict the occurrence of caloric or nutritive outcomes(33). In fact, the sweet taste receptors exist at the level of the taste buds on the tongue. Once they perceive the sweet taste, they induce downstream activation of second messenger systems and signals the hypothalamus that controls the thermoregulation. The brain is able to detect and predict the energy-yielding from the food consumed. When sucralose and aspartame are consumed, the brain perceives that there are sweet and energetic compounds entering the GI tract; thus, the brain increases the efficiency of nutrient utilization and creates a positive energy balance. This is known as the cephalic-phase response. The increase in body weight and fat mass that resulted from aspartame intake and then sucralose intake could be associated with the alteration of the cephalic-phase response to the ingested food; thus the perturbation in the thermoregulation. Swithers et al. claimed that in response of the activation of sweet taste receptors by the AS saccharine, the thermic response to food was blunted; thus the thermoregulation was affected resulting a positive energy balance

that was associated with an increase in body weight, increase in adiposity, and decrease in energy expenditure.(33). This mechanism could explain the increase in body weight and the alteration in body composition. To the best of our knowledge, there aren't any recent studies that assessed the association between aspartame and sucralose intake with the thermoregulation and energy expenditure. In addition to that, Mitsutomi et al. demonstrate that AS resulted in a positive energy balance that is not related to the brain but to the adipocytes. The brown adipose tissue is a non-shivering thermogenic organ that is involved in energy metabolism. The uncoupling proteins 1 are expressed in brown adipose tissues, and they are responsible for the thermogenic capacity of the brown adipose tissue because they are mitochondrial transporters, they act as proton carriers; thus, they are involved in the respiratory chain and heat production. Several studies have confirmed the association between the uncoupling protein 1 and body weight, through its involvement in resting energy expenditure and substrate oxidation. Mitsutomi et al. fed obese mice AS which resulted in a decrease in uncoupling protein, followed by an increase in adiposity, increased body weight, and glucose intolerance. However, they did not specify what kind of AS they used in their study(16). Moreover, the sweet taste receptors exist at the level of the GI tract, once they are activated, the endocrine cells secrete incretine hormones; thus, the release insulin gets stimulated. In fact, when aspartame and sucralose are consumed, they activate the sweet taste receptors in the intestine, the incretine hormones GLP-1, PYY, and glucose-dependent insulinotropic peptide(GIP) are secreted and they stimulate insulin secretion. The insulin is released but does not find a nutritive sweet compound to metabolize it. Also, the pancreatic ß- islets cells have sweet taste receptors, in case of aspartame and sucralose consumption, they receive a signal that a sweet compound needs to be

metabolized. This compound is expected to be a sugar. Therefore, insulin is released but does not find sugar to metabolize it. This process affects the insulin sensitivity; it exhausts the pancreatic ß cells. Prolonged intervention could result in further deterioration of pancreatic ß cells, leading to metabolic disturbances or exacerbating the situation in case the consumers are already suffering from metabolic illnesses. For instance, Shastry et. al divided their interventional rat study into three phases depending on the dose and the time (they increased dose), the negative effects of sucralose and aspartame on glucose tolerance started to appear on phase 2 (3-7 weeks of intervention). However, Shastry et. al used clinically insignificant doses of aspartame and sucralose that usually a person cannot reach in real life, they begun at phase 1 with the acceptable daily intake set by FDA for 3 weeks, phase 2 starting from week 3 until week 7 with a dose 2 times higher than the acceptable daily intake, and phase 3 starting from week 7 until week 13 with a dose 4 times higher than the acceptable daily intake(37). Furthermore, there is another proposed theory that correlates aspartame consumption to glucose intolerance that includes the intestinal alkaline phosphatase (IAP). IAP has been associated with metabolic syndrome and glucose intolerance. One of the breakdown products of aspartame is phenylalanine that inhibits IAP. Gul et al. indicated that mice that consumed a combination of high fat diet with aspartame-sweetened fluid had an inhibition in IAP, followed by a significantly higher body weight, higher glycemia, and glucose intolerance when compared with mice fed high fat diet with regular water(38).

The insulin sensitivity and the pancreatic ß cells functions were assessed using the HOMA formulas. We did not observe any significance in the results. However, there are many controversies regarding the validation of these formulas. Some studies suggest that HOMA formulas were invented to predict the sensitivity of insulin and the

function of pancreatic cells among humans, and they can't be used on animals; however, other studies validated the HOMA formula usage on rats. Therefore, it is preferable to use the gold standard hyperinsulinemic-euglycemic clamp; but unfortunately, it is not cost-effective. Our study findings are similar to the rodent study of Sanchez-Tapia et al. that showed that sucralose was associated with a higher weight gain, and a higher rate of lipogenesis. Additionally, the study revealed that the rats fed sucralose had higher serum glucose when compared to the consumption of glucose, other natural sweeteners, or the absence of any sweetener. The combination of high fat diet with sucralose increased more the blood glucose. This finding is interesting because AS are a characteristic of the westernized dietary pattern that is characterized by high fat food. After 4 months of sucralose consumption, the rats developed hyperinsulinemia as well, even more than the rats fed sucrose did. This outcome is similar to our study, although the difference did not reach significance; but we believe longer duration of the study similar to Sanchez-Tapia et al. study could have shown a statistically significant outcome. Sanchez-Tapia et al. suggested that sweet taste receptors are also present at the level of adipose tissues, this is another reason why AS are influencing the body composition and body weight. In their study, sucralose was found to be associated with adipocytes. In fact, rats fed sucralose had a big size of adipocytes, and they had hyperleptinemia (leptin is secreted by the adipocytes). When the AS bind to the sweet taste receptor at the level of the GI tract, the incretine hormone GIP is secreted and it binds to its receptor on the adipose tissues (both brown and white). GIP plays an important role in the metabolism of adipose tissue. The increase of GIP by sucralose affects the insulin sensitivity(36).Another remarkable finding by Sanchez-Tapia et al. is that the negative metabolic effect of sucralose was independent of the dose, which is

contrary to our outcomes. In our study, it was noticeable that when the dose of sucralose or aspartame increases, the effect becomes more pronounced, although it did not reach significance. We believe longer duration of the intervention could have shown a significant difference. Also, the incretine hormones like GIP have receptors at the level of adipose tissues, and they regulate the proinflammatory action. Inflammation is associated with a decrease in insulin sensitivity and an increase in blood glucose. This could be another explanation for the hyperinsulinemia and hyperglycemia that resulted from aspartame and sucralose intake in our study. In their review, Fowler et al. proved that aspartame and sucralose have an impact on the metabolism, the body weight, the blood glucose, the insulin sensitivity, and the cardiometabolic risk based on animal studies and human studies. Interestingly, fowler et al. stated that there are many studies available in the literature that investigated the metabolic effects of aspartame and sucralose; nevertheless, their effects varied with intrinsic characteristic of the study and the study population. In fact, Fowler et al. determined that multiple factors modulate the effects of AS on health outcome (gender, genetic predisposition, diet adapted, metabolism, cardiometabolic conditions dose of AS and timing of initiation of exposure). In human studies, it is difficult to control all these factors; but in our rodent study those factors are well controlled(17).

In addition, research studies have shown that the gut microbiota consists of around 160 bacterial species that are involved in the human metabolism. In fact, they contribute enzymes that are not encoded by the human genome such as the fermentation of indigestible carbohydrates and the synthesis of vitamins. Also, they are involved in the metabolism of carbohydrates, protein, and fat; thus, its composition is associated with the pathogenesis of metabolic syndrome, obesity, insulin resistance, and even type

two diabetes. Unfortunately, aspartame and sucralose alter the gut microbiota. They were shown to have a bacteriostatic effect, that remained for 3 months after cessation of consumption(12). A study on Sprague Dawley rats has demonstrated that 1.1–11mg/kg/d of sucralose resulted in an alteration in the gut microbiota composition, which is similar to the amount of sucralose ingested in our interventional rat study(39). Sucralose decreases intestinal bacteria, its effect is more pronounced on the beneficial bacteria since it suppresses the beneficial anaerobes (*Lactobacilli* and *Bifidobacteria*), and does not inhibit the detrimental bacteria (enterobacteria). Sucralose perturbates the entire bacterial ecosystem of the GI tract, and it remains for months after recovery(9). Furthermore, 5-7 mg/kg/d of aspartame induced significant changes in the gut microbiome. When the aspartame intake is accompanied with a high fat diet, the negative effect on gut microbiota becomes more pronounced(40).

Research found a difference between the gut microbiota of lean individuals and obese individuals. Gram (-) *Bacteroidetes* and Gram (+) *Firmicutes* are the most dominant bacteria in the gut. An increase in *Bacteroidetes* is associated with healthy weight, it is found abundant in the gut of lean individuals. Whereas, an increase in *Firmicutes* is associated with obesity, it is abundant in the gut of obese individuals and consumers of AS. Consumers of AS have a high ratio of *Firmicutes/ Bacteroidetes*(9). Aspartame and sucralose are bacteriostatic, they were found to be associated with dysbiosis and endotoxemia that result in an inflammatory response and insulin resistance. In fact, sucralose and aspartame inhibit the growth of bacteria, alter the gut microbiota, yielding an increased release in lipopolysaccharide (LPS). LPS will be absorbed into the circulation, it will bind to CD14 proteins (responsible for modulation of insulin sensitivity in case of hyperglycemia, hyperinsulinemia, and weight gain),

nucleotide oligomerization domains (NODs), and Toll-like receptors (TLRs) on the surface of the immune cells macrophages and dendritic cells. The activation of the immune cells will result in a release of cytokines and an inflammatory response. Further signaling pathways in metabolic cells will be activated, and it will lead to insulin desensitization, impaired expression of proteins that transport glucose, increased intestinal permeability, oxidative stress and inflammation of adipose tissues. Therefore, it is hypothesized that the alteration of gut microbiome composition by aspartame and sucralose could be another reason to explain the mechanism behind the negative metabolic effects of aspartame and sucralose.

In addition, it is worth noting that sucralose is an organochlorine sweetener. Extensive research studies have found that the exposure to organochlorine compounds (used in pesticides) is associated with increased weight gain, obesity, and diabetes. Therefore, the approval for the usage of sucralose, the organochlorine compound as a safe artificial sweetener should be reconsidered.

On the other hand, there wasn't a difference in triglyceride and VLDL levels between the sucralose groups and the control group. There are limited research studies that assessed the effect of sucralose on triglycerides. The main focus of the literature is on the effect of sucralose on body weight, glucose and insulin tolerance, since the AS are used mostly by obese and diabetics in order to replace the caloric sugar, reduce weight and improve the glycemia. But, sugar is associated with hypertriglycedemia, and AS may be used by individuals to replace sugar and lower triglyceride and VLDL levels. The outcome of our study is similar to previous animal and human research studies that demonstrated that sucralose does not have an impact on triglyceride level, regardless of the dose of sucralose and the metabolic status of consumers(41-43). Ibero

et al. conducted a randomized controlled trial about the difference in metabolic effects of a sucralose-sweetened jam vs. sugar-sweetened jam. Although there wasn't a significant difference between serum TG of the group consuming sucralose-sweetened jam vs. the group consuming sucrose-sweetened jam; however, postprandial free fatty acid (FFA) in plasma were greater among the group of sucralose-sweetened jam than the group of sucrose-sweetened jam. FFA in blood lead to insulin resistance. This indicates that sucralose perturbed lipid metabolism(44). By contrast, in their rat study, Saada et al. claimed that sucralose decreases TG because it has an effect on peroxisome proliferator-activated receptors-alpha (PPARs-α); therefore, it increases the expression of lipoprotein lipase. Also, PPARs stimulates triglycerides storage(45). This could possibly explain the change in body composition of the rats administered sucralose in our study. On the contrary, aspartame increased triglyceride levels when compared with the control group and sucralose groups. When the dose of aspartame increases, triglyceridemia increases; but it did not reach a statistically significant difference. It is hypothesized that consumption of aspartame for a longer period of time could develop a negative effect on triglyceride levels. Aspartame is a major ingredient in sugar-free carbonated beverages that are proven to be associated with metabolic syndrome(27).Lebda et al. demonstrated in a rat study that replacing nutritionally sweet soft drinks with aspartame is not effective(27). Aspartame is associated with an increased in triglyceride and VLDL compared to control (water). It reduced PPARs-γ, thus reduced LPL activity, resulting in an accumulation of FFA and hypertriglyceridemia. Also, aspartame increased hepatic enzymes alkaline phosphatase and aspartate aminotransferase reflecting hepatic damage and necrosis. The hepatic histology analysis indicated an inflammatory response in the liver. Aspartame also

induced oxidative stress, it is associated with an increase in lipid peroxidation because it altered the endogenous antioxidant system since it significantly reduced the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, and reduced glutathione when compared with the control group. Lebda et al. stated that the oxidative stress resulted from aspartame intake is due to its metabolite methanol that is a toxic and carcinogen product. However, Marinovich et al. indicated that methanol is a compound found naturally in food and the average consumption of aspartame does not reach a level that produces a toxic amount of methanol. For instance, a cup of tomato juice produces 6 times more methanol than a can of aspartame-sweetened beverage(46). But, it is theorized that chronic consumption of aspartame that is broken down to methanol could yield a toxic and unhealthy effect. Fruits are nutritious and complex, but an aspartame-sweetened beverage like Coca-Cola are basically made of phosphoric acid, aspartame, and caffeine. Therefore, their content is different, their digestion and metabolism will be different. Furthermore, it is not only methanol its self that is toxic, it is also formate a metabolite of methanol (Aspartame→ Methanol→ Formaldehyde→ Formate). The negative effect of aspartame on oxidative stress could be another possible explanation for the insulin resistance that started to develop in our study, because oxidative is associated with insulin resistance. It is worth noting that Lebda et al. used a clinically insignificant amount of aspartame that does not mimic real life situation (240 mg/kg body weight/day orally equivalent on average to 72 g/day). Prokić et al. assessed the effect of aspartame (40 mg/kg/daily for six weeks, less than the ADI; which is equivalent to 12g/day for 6 weeks) on lipid profile and oxidative stress. The interventional amount of aspartame used in their study is less than the one used by Lebda et al. but still higher than the amount used in our study. They also found that

aspartame is associated with oxidative stress, permeability, damage, and necrosis of hepatocytes(34). Therefore, according to the literature, aspartame damages the liver that plays a major role in lipid metabolism(43). This could explain the increase in TG and VLDL level among aspartame consuming rats comparing to rats fed starch without AS.

Furthermore, there wasn't a significant difference in total cholesterol, HDL, and LDL levels between all the groups (the control group without AS, aspartame groups, and sucralose groups). Nevertheless, the lowest level of HDL was found among the rats fed the highest amount of sucralose, LDL was not affected on the contrary, it was less than the control group but the difference is not statistically significant. HDL is responsible for uptake of cholesterol from tissues to the liver. Decreased level of HDL is associated with metabolic diseases. The alteration in HDL levels among the rats fed highest amount of sucralose could be a consequence of their increase in body weight and body fat mass. Also, it could be a result of liver damage like previous studies have shown, since the liver plays a major role in HDL metabolism (synthesis of Apo protein A-I and enzymes such as cholesteryl ester transfer protein (CETP), lecithin: cholesterol acyltransferase (LCAT), hepatic lipase (HL) and protein phospholipid transfer protein (PLTP). In rats fed aspartame, the HDL decreased slightly when the dose of aspartame increases; however, it did not reach a statistically significant difference, and the decrease was more remarkable among the rats fed sucralose. In spite of that, aspartame intake was associated with a small increase in LDL level when compared with the control group or sucralose group, but it was not statistically significant. Our findings contradict with other studies that demonstrated that aspartame alters the lipid profile (increase in total cholesterol, increase in LDL, and decrease in HDL) and the liver function. Chronic consumption of aspartame could result in metabolic syndrome and

atherosclerosis(27, 34). Also, based on a rat study, aspartame might exacerbate the lipid profile of metabolically ill cases compared with healthy and non-consumers of aspartame. The finding is alarming because aspartame is used among diabetic to lower the diabetes complications(43).

In addition, albumin, creatinine and BUN levels were normal, there wasn't any statistically difference between the control group, aspartame groups, and sucralose groups. Therefore, sucralose and aspartame did not damage the kidneys and affected their function. Also, the normal levels of albumin and creatinine in aspartame groups and sucralose indicate that the liver is not damaged and it is functioning. However, it is better to rely on liver histology analysis and liver enzymes assessment in order to evaluate the function of the liver. Prokić et al. demonstrated that aspartame intake altered the liver function and the liver enzymes while albumin remained normal(34). Our results contradict with the study of Helal et al. that indicated in their rat study that sucralose consumption (5 mg/kg/day) increases serum urea and creatinine and decreases serum albumin; thus the sucralose affected the function of the liver and the kidneys(47). Sucralose is an amphiphilic organochlorine compound; thus, it bioaccumulates in organs such as kidneys and can cause damage. Clearly the highest level of BUN in our study is among the rats fed highest level of sucralose. Although it is not statistically significant different from the control group and the aspartame groups; but, it could reflect that sucralose started to affect the kidneys function. longer duration of exposure to sucralose could have demonstrated significant effects on the kidneys. Additionally, the lowest level of creatinine in our study is found among the rats that consumed highest level of aspartame. Although the difference between creatinine levels among the aspartame groups and control and sucralose groups is not significantly different;

however, this could indicate a beginning of a malfunction in the liver since the liver metabolizes creatine into creatinine.

Furthermore, Sucralose and aspartame did not affect the weight of heart, kidneys, and liver since there wasn't a statistically significant difference between all the groups. On the contrary, Goldsmith et al. showed that sucralose affects organ weight in rats (heart, lungs, liver spleen, thymus, ovaries, prostate, adrenals, and caccum) for chronic use (8 weeks). Also, acute intake of sucralose for 4 weeks affected the organ weights (heart, spleen, thymus, prostate, caccum, brain, uterus, testes). The toxic effects depend on the gender of the rats and on the dose of sucralose. But the dose used in the study is a toxic level and administered by oral gavage(48). In our study, we used adequate amounts in food and water for 8 weeks, and they didn't affect the organs; therefore, moderate amounts of sucralose and aspartame are safe, they don't have toxic effects. However, it is believed that longer duration of consumption could possibly affect the organs and develop toxic and carcinogenic side effects since sucralose was shown to bioaccumulate in organs such as kidneys and adipose tissues, and aspartame was shown to be metabolized into methanol and formate, toxic and carcinogenic compounds. More research should investigate the effect of chronic and moderate consumption of aspartame and sucralose on the metabolism. Moreover, the percentage of epididymal weight of rats fed highest amount of aspartame in food and water is significantly higher than the control, and the group fed lowest amount of sucralose in water only. Therefore, aspartame is associated with an increase in total body fat (since total body weight and the fat mass increased), accompanied with an increase in visceral fat associated with metabolic diseases, diabetes, and cardiovascular diseases. Aspartame is obesogenic, and when the dose increases the fat increases until it accumulated

between the organs. This outcome also validates our hypothesis that aspartame is associated with insulin resistance and glucose intolerance. Sucralose might have a more attenuated effect on obesity than aspartame; however, it is important to note that in our study the dose of sucralose was less than the dose of aspartame because sucralose is sweeter than aspartame.

Sucralose and aspartame were tested for safety but limited work has been done on their efficacy.

CHAPTER VI

CONCLUSION

In conclusion, AS were introduced to the market to replace sugar and decrease the burden of obesity and its related metabolic disorders. However, the intended effects of AS do not seem to correlate with what is observed in clinical practice. AS have disastrous impacts on the metabolism. More long-term research studies are needed, targeting humans and special populations (pregnant women, children, diabetics...). The research findings show that it is urgent to raise public awareness and governmental responses for policies and regulations targeting the potential harmful health effects of excessive and/or chronic consumption of AS, particularly on weight and metabolism. Sucralose and aspartame are calorie free, but at what health cost?

APPENDICES

Appendix 1

***** Prior to the 2 days of sacrifice, preparations have been made:

1. Categorization of rats based on their weight, in a descending order.

2. Dividing rats into two groups to sacrifice them. Group 1 sacrificed on day 1 of sacrifice. Group two sacrificed on day two of sacrifice.

3. Labelling of rat cages based on the order of sacrifice (rat with the highest weight will be sacrificed first).

4. Preparing of the materials needed for the sacrifice and the collection of blood and organs:

- Ethylenediaminetetraacetic acid (EDTA) tubes labelled with the number of rat (3 tubes for each rat). These tubes are used because they contain an anticoagulant important to perform plasma analysis. EDTA binds the calcium ions and therefore blocks the coagulation cascade
- Chloroform tubes labelled with the number of rat. These tubes are used because they preserve the hepatic tissues, in order to perform a histological analysis.
- Labelled aluminum cuts and nylon bags with the number of rat and the organ collected.
- Ice box and crushed ice to put the blood tubes inside immediately after collection and before storage in the freezer
- Liquid nitrogen to preserve the organs immediately after collection and before storage in the freezer

- Dissection tools: scissors, and iris tissue forceps.
- Disposable hypodermic needle 20G x 1 $^{1/2}$
- BD Syringe 10 ml
- Anesthetic mask
- Inhalation anesthetic isoflurane (Forane®, 134 Abbott, Berks, UK)
- Container containing cotton and filled with anesthesia in order to put the rat inside.
- Face mask, gloves, shoe covers, hairnets, and lab garments.
- Ethanol
- Paper roll and disposable tissues
- Scale to weight the fasting weight of rats, and to measure the weight of the organs collected
- Betadine, in case of accidents while manipulating, it can be directly applied to the injured researcher

Appendix 2

** The following is the procedure performed to analyze insulin via the ELISA technique at the nutrition laboratory, Faculty of Agriculture and Food Sciences-American University of Beirut:

1. Preparation of the material required for ELISA:
    - Calibrated Pipettes with tips, 10 µL-80 µL
    - Multi-channel Pipettes with tips, 300 µL
    - Reagent reservoir
    - Vortex mixer
    - De-ionized water
    - Bechers
    - Microtiter Plate Reader capable of reading absorbency at 450 nm and 590 nm
    - Orbital Microtiter Plate Shaker
    - Absorbent disposable tissues
    - Adhesive Plate Sealer
    - Plasma samples
    - ELISA plate coated with anti-rat insulin antibodies
    - The reagents obtained with the ELISA kit, pre-warmed at the room temperature.
2. Assay procedure
    i. Preparation of the Wash Buffer concentrate by mixing the solution with 900 ml de-ionized water inside a labelled Becher.

ii. Washing each well with 300 µL Wash Buffer concentrate by using a multi-channel Pipette with tips, and then taping the plate onto absorbent disposable tissues to remove any residues. The step is repeated 3 times. Disposing the tips in a Becher, and then throwing them in a hazardous bag.

iii. Adding 10 µL of the Assay Buffer to the Blank wells (without insulin) and the sample wells using a 10 µL pipette with strips. Disposing the tips in a Becher, and then throwing them in a hazardous bag.

iv. Adding 10 µL of the Matrix solution to the blank, standard, and control wells. Disposing the tips in a Becher, and then throwing them in a hazardous bag.

v. Adding in duplicate 10 µL of the Rat Insulin Standard solution to all the wells. There are 6 standard insulin concentration (0.2- -0.5- 1- 2- 5- 10 ng/mL), it should be added to the wells appropriately and in an ascending order. Removing the tips when the insulin standard solution is changed. Disposing the tips in a Becher, and then throwing them in a hazardous bag.

vi. Adding 10 µL of the Quality Controls 1 and 2 solutions to the appropriate wells. Disposing the tips in a Becher, and then throwing them in a hazardous bag.

vii. Using a vortex mixer, the blood samples are mixed and homogenized. Adding in duplicate 10 µL of the blood samples into the remaining wells. Disposing the tips in a Becher, and then throwing them in a hazardous bag.

viii. Adding 80 µL of the Detection Antibody solution to all the wells. Disposing the tips in a Becher, and then throwing them in a hazardous bag.

ix. Covering the plate with a labelled adhesive plate sealer.

x. Incubating the plate at room temperature for 2 hours, on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 Revolutions Per Minute (rpm).

xi. Removing the adhesive plate sealer. Decanting the plate content while taping it to remove any residues.

xii. Washing each well with 300 µL Wash Buffer concentrate by using a multi-channel Pipette with tips, and then taping the plate onto absorbent disposable tissues to remove any residues. The step is repeated 3 times. Disposing the tips in a Becher, and then throwing them in a hazardous bag.

xiii. Adding 100 µL of Enzyme solution to each well. Disposing the tips in a Becher, and then throwing them in a hazardous bag.

xiv. Covering the plates again with a labelled adhesive plate sealer.

xv. Incubating for 30 minutes on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 Revolutions Per Minute (rpm).

xvi. Removing the sealer. Decanting the plate content, and then taping the plate onto absorbent disposable tissues to remove any residual fluids.

xvii. Washing each well with 300 µL Wash Buffer concentrate by using a multi-channel Pipette with tips, and then taping the plate onto absorbent

disposable tissues to remove any residues. The step is repeated 6 times. Disposing the tips in a Becher, and then throwing them in a hazardous bag.

xviii. Adding 100 µL Substrate solution to each well. Disposing the tips in a Becher, and then throwing them in a hazardous bag.

xix. Covering the plates again with a labelled adhesive plate sealer

xx. Incubating for 5-20 minutes on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 Revolutions Per Minute (rpm). A blue color is formed into the standard wells, with an intensity proportional to increasing concentration of insulin.

xxi. Removing the sealer. Adding 100 µL Stop Solution. Disposing the tips in a Becher, and then throwing them in a hazardous bag.

xxii. Shaking the plate by hand to ensure complete mixing of solution in all wells. The blue color should turn into yellow after acidification.

xxiii. Placing the plates inside the ELISA machine "Thermo Scientific Multiskan Go". Reading the absorbance at 450 nanometer (nm) and 590 nm in a plate reader within 5 minutes and ensure that there aren't air bubbles in any well. Recording the difference of absorbance units.

3. Calculation of insulin

For sample interpretation, a graph is plotted as a reference curve using the values of absorbance unit of 450nm, less that of 590nm of the rat insulin standards. On the Y- axis, the absorbance unit, and on the X-axis the concentration of rat insulin standards.

The ELISA was successful and the values were acceptable, since all the values of the Quality Control samples fell within the calculated Quality Control Range.

Appendix 3

***The following is the procedure followed to assess the serum metabolic markers:

1. Preparation of the machine
2. Placing the control samples to assess the specificity of the machine. Placing the cartages of the required tests inside the machine. The results obtained were within the standardized normal range; thus, the machine is well calibrated and the results are specific.
3. Using a vortex mixer, the serum samples are mixed and homogenized. Using a 300 μL pipette to extract serum and placing it in the tray. Changing tips between each blood sample. Disposing the tips in a Becher, and then throwing them in a hazardous bag.
4. Placing the tray with serum samples inside the machine
5. Collecting results from the screen

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