Quercetin as a prophylactic against adverse health outcomes associated with high dietary fructose post-weaning in Sprague Dawley rats

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Declaration

I, Ramatsobane Rozette Tladi, hereby declare that this dissertation is my own work, with the assistance of the acknowledged persons. It is being submitted for the degree of Master of Science in Medicine in the Faculty of Health Sciences in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University. All procedures used in this dissertation were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC number: 2017/02/07B).

Tladi R.R.

Signed on the 26 day of August in 2020

Dedication

To my parents and little brother whom I admire and adore. Thank you for your unwavering support. I hope I make you proud.

Conference presentation

The following poster was presented at the **47th conference of the Physiology Society of Southern Africa** hosted by Walter Sisulu University at the ICC, East London, from the **18th - 21st August 2019**:

Tladi R.R., Erlwanger K.H. and Donaldson J. Effect of quercetin on the development of non-alcoholic fatty liver disease and dyslipidaemia in Sprague Dawley rats fed a high fructose diet, post-weaning.

Abstract

The global increase in fructose consumption has increased the prevalence of metabolic syndrome (MetS) and non-alcoholic fatty liver disease (NAFLD). Events affecting metabolism in early life have long-lasting implications on health outcomes later in life. Thus, the developmentally plastic early life stages are potential targets for interventions with long term health benefits. The prophylactic potential of the phytochemical quercetin administered during the early post-weaning period, on the development of metabolic derangements and NAFLD in Sprague Dawley rats, fed a high fructose diet for eight weeks post-weaning was investigated. Rats (N = 64; 21 days old) were randomly divided into four treatment groups n = 16 (8 males and 8 females): Group 1-standard rat chow (SRC), tap water, plain gelatine cubes; Group 2- SRC, tap water, gelatine cubes containing quercetin (100 mg/kg body mass); Group 3- SRC, 20 % fructose drinking solution (FS), plain gelatine cubes; Group 4- SRC, 20 % FS, gelatine cubes containing quercetin (100 mg/kg body mass). Rats received treatments for two weeks, after which they continued on their respective diets without gelatine cubes for six weeks. Tissue and blood samples were then collected and analysed for fructose induced metabolic derangements. Male rats fed fructose had significantly shorter and lighter tibia and femora compared to those in the control group ($p < 0.05$). Although no statistically significant differences ($p>0.05$) in the overall hepatic steatosis scores were observed between groups in male and female rats, a greater percentage of male rats fed a high fructose diet presented with steatosis. There was also an increased percentage of female rats with steatosis after being fed quercetin alone. Furthermore, there were no significant changes in liver function markers, adiposity, circulating metabolites, lipoprotein profile and clinical markers of general health ($p > 0.05$). Despite the experimental model not resulting in overt metabolic dysfunction, the sexually dimorphic susceptibility to the development of hepatic steatosis and the impact of early post-weaning quercetin, should be further explored.

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List of Abbreviations

6-PGDH: 6-phosphogluconate dehydrogenase

ACC: Acetyl-CoA Carboxylase

AESC: Animal Ethics Screening Committee

ALT: Alanine transaminase

ANOVA: Analysis of variance

apoA-I: Apolipoproteins A-I

apoA-II: Apolipoproteins A-II

BUN: Blood urea nitrogen

C: Control group

CCE: Citrate cleaved enzyme

Crea: Creatinine

DOHaD: Developmental origins of health and disease

F: Fructose group

F+Q: Fructose + Quercetin group

FAS: Fatty Acid Synthetase

FS: Fructose drinking solution

GLUT5: Glucose transporter-5

HDL: High-density lipoproteins

HO-1: Heme oxygenase-1

ME: Malic enzyme

MetS: Metabolic syndrome

NAFLD: Non-alcoholic fatty liver disease

Nrf-2: Nuclear factor erythroid 2-related factor 2

OD: Optical density

PBF: Phosphate buffered formalin

Q: Quercetin group

SRC: Standard rat chow

SREBP-1: Sterol Regulatory Element-Binding Proteins 1

T2D: Type 2 diabetes mellitus

TGs: Triglycerides

VLDL: Very low-density lipoprotein

WHO: World Health Organisation

CHAPTER 1: LITERATURE REVIEW

1.1 Metabolic syndrome

1.1.1 Definition

Metabolic syndrome (MetS) is a group of conditions which are considered to be cardiovascular and type 2 diabetes mellitus (T2D) risk factors, these include, obesity (particularly visceral obesity), glucose intolerance, dyslipidaemia (i.e. hypertriglyceridemia, increased free fatty acids and decreased high-density lipoprotein cholesterol (HDL-C), microalbuminuria and hypertension (Buzzetti et al., 2016; Falahi et al., 2015). Over the years, there have been several criteria used to diagnose MetS. In 1998 the World Health Organisation (WHO) was the first organisation to define MetS (Balkau and Charles, 1999). In 1999 the European Group for the study of Insulin Resistance proposed a modified definition to that of the WHO that excluded individuals that were already diagnosed with T2D as having MetS. In 2005, the International Diabetes Foundation issued a new criterion for MetS which did not necessitate insulin resistance to be present. In 2001 the National Cholesterol Education Program Adult Panel III presented a definition for MetS which was updated by the American Heart Association and National Heart, Lung and Blood Institution in 2005 which has since become one of the most commonly used criteria of MetS (Grundy et al., 2005; Huang, 2009).

According to the National Cholesterol Education Program Adult Treatment Panel III, a MetS diagnosis is made when someone has three or more of the following criteria, abdominal obesity: waist circumference > 102 cm in men and >88 cm in women; hypertriglyceridemia: ≥ 150 mg/dL (1.67 mmol/L); low HDL-C: <40 mg/dL (1.04 mmol/L) in men and < 50 mg/dL (1.29 mmol/L) in women;. high blood pressure ≥ 130/85 mmHg; high fasting glucose ≥ 100 mg/dL (≥ 5.6 mmol/L) (Kelliny et al., 2008; Saklayen, 2018).

1.1.2 Prevalence of MetS

The global prevalence of MetS, as defined by the WHO, ranges between 7.4 % and 50 %, this accounts for over a billion people in the world (Falahi et al., 2015; Saklayen, 2018). In recent years the prevalence of non-communicable diseases in African countries has increased and is projected to supersede the prevalence of communicable, maternal, perinatal, and nutritional diseases as the most common

cause of death by 2030 (Holmes et al., 2010; Tran et al., 2011). Countries in Africa, such as Nigeria, Seychelles, Botswana and Cameroon have reported an escalation in the prevalence of non-communicable diseases since the beginning of the century (Okafor, 2012). The rapid increase in the prevalence of MetS in recent years has been attributed to urbanisation, consumption of westernised diets and physical inactivity (Owolabi et al., 2017).

The main features of MetS include dyslipidaemia, insulin resistance, T2D, hypertension and central obesity (Felice et al., 2014). Management of these diseases is associated with high costs (Marangos et al., 2010). There is lack of information on the costs of managing MetS as a syndrome, however information which is available, is for the specific risk factors. For example, between 2003 and 2014, the costs associated with management of hypertension in the United States were estimated to be US$ 131 billion of national health care expenditure averaged over 12 years (Kirkland et al., 2018). In recent years the healthcare costs have shifted from hospitals and central governments to individual patients bearing the economic burden of the disease (Alcocer and Cueto, 2008). Sub-Saharan African countries spend an average of 6 % of their Gross Domestic Product on healthcare (Plewes and Kinsella, 2012). There is a wide range in healthcare expenditure, for example, in South Africa US$ 390 was spent per person on health care which is high compared to US$ 6 spent in Ethiopia (van de Vijver et al., 2013).

The International Diabetes Foundation estimates that Africa spends about 7 % of its healthcare budget on diabetes. It has also been reported that the number of people diagnosed with diabetes in Africa will increase from 14.2 million to 34.2 million by 2040 (International Diabetes Federation, 2015). In 2015, the overall cost of diabetes treatment was US$ 19.45 billion, which accounted for 1.2 % of Sub-Saharan African regions gross domestic product. In South Africa, in 2018, the government sector spent ZAR 2.7 billion on T2D patients which accounted for 1.5 % of the health departments' annual budget (Erzse et al., 2019).

The risk factors for MetS are also closely related with non-alcoholic fatty liver disease (Bruce and Hanson, 2010; Buzzetti et al., 2016) which also places an immense burden on healthcare delivery.

1.2 Non-alcoholic fatty liver disease

1.2.1 Definition

Non-alcoholic fatty liver disease (NAFLD) is a sweeping term that includes conditions like simple steatosis, steatohepatitis, liver fibrosis and cirrhosis (Buzzetti et al., 2016; Cohen et al., 2011; Younossi et al., 2016). NAFLD is defined as the presence of steatosis with ≥ 5 % fat infiltration in imaging or histology, in the absence of alcohol consumption, drugs that induce steatosis or competing liver disease aetiologies such as chronic viral hepatitis and viral induced steatosis (Buzzetti, Pinzani and Tsochatzis, 2016; Mishra and Younossi, 2012).

Mild and asymptomatic elevation of liver enzymes are initial indicators for the presence of NAFLD (Grander et al., 2016). Patients are generally fatigued, with right upper quadrant discomfort. Even though liver enzyme concentrations may change overtime, patients with advanced liver disease (NAFLD) can have normal liver enzyme concentrations (Fazel et al., 2016). It is also reported that NAFLD patients with T2D and older age are at higher risk for having fibrosis (Mishra and Younossi, 2012).

1.2.2 Prevalence of NAFLD

Despite limitations in the screening for NAFLD, owing to low accuracy of non-invasive screening tools (Paschos and Paletas, 2009), studies suggest that the global prevalence of NAFLD is 25.24 %, with Africa having the lowest prevalence while Middle Eastern and South American countries have the highest prevalence (Younossi et al., 2016). This disease is also found in young people, with between 2.6 % and 9.8 % of children and adolescents being affected (Pacifico et al., 2011). Obese individuals make up the largest proportion of people affected by NAFLD, with 75 % of the people with NAFLD being obese, compared with just 16.5 % of the affected population being lean (Paschos and Paletas, 2009). Studies conducted in South Africa found that 36 % of the individuals diagnosed with NAFLD on biopsy had non-alcoholic steatohepatitis, of which 17 % had fibrosis (Spearman and Sonderup, 2015).

Data on the costs of managing NAFLD are lacking for most developing countries, however, in the USA it was estimated that it cost US$ 103 billion, which was high compared to European counties where it was € 27.7 billion in France, Italy and Germany collectively (Younossi et al., 2016). Therefore, like diabetes mellitus, NAFLD burdens healthcare facilities.

1.2.3 Causes of NAFLD

NAFLD is closely related to components of MetS specifically insulin resistance, dyslipidaemia and obesity, with visceral obesity considered as a risk factor for the development of NAFLD (Grander et al., 2016). NAFLD is common in patients with dyslipidaemia particular those with high triglyceride (TGs) levels and low serum HDL-C (Chalasani et al., 2018). There also seems to be a genetic risk to development and progression of the disease, with individuals with family history of T2D being at increased risk of progression to Non-alcoholic steatohepatitis (Fazel et al., 2016). However, recent evidence shows a positive correlation between added dietary sugar (particularly fructose) and NAFLD, MetS and weight gain (Dinicolantonio et al., 2017).

1.3 Fructose: A common causal agent in MetS and NAFLD

1.3.1 Fructose consumption

Fructose is found in fruits, vegetables and honey. In recent years the bulk of fructose ingested is provided by commercially produced products such as high-fructose corn syrup and sweetened beverages (Kelishadi, Mansourian and Heidari-Beni, 2014). High fructose consumption has been related with increased adiposity, obesity, oxidative stress, inflammation and subsequently disorders in insulin function (Pereira et al., 2017). The overall sugar consumption (fructose) of South African school aged children via the consumption of beverages and processed food was reportedly high, at 84.1 g/day, compared to consumption observed from other African countries, such as Kenya (50.1 g/day), Malawi (24.1 g/day) and Uganda (25.7 g/day) (Steyn and Temple, 2012). This increased consumption of fructose in South Africa has been reported to be associated with the high incidence of obesity and its complications (Prinsloo et al., 2011; van der Merwe and Pepper, 2006). According to recent reports, South Africa was recorded to have the highest prevalence of obesity on the continent (57 % in women and 29 % in men), compared to 10 % in the West Africa region

(Abubakari et al., 2008). In this regard there have been discussions and introduction of sugar tax (Du et al., 2018; Manyema et al., 2016) as a concerted effort by government to reduce the sugar content of beverages (Allcott et al., 2019).

In order to understand why fructose is such a health problem, it is important to consider its metabolism.

1.3.2 Fructose metabolism

Glucose transporter-5 (GLUT5), a fructose specific transporter, is highly expressed along the small intestine brush border, and GLUT2, which transports both fructose and glucose is expressed in the liver, small intestine (along the basolateral membrane), and pancreas (Cheeseman, 1993). The majority of dietary fructose is transported via the portal circulation to the liver (Herman and Samuel, 2016). Contrary to other monosaccharides, fructose does not require ATP hydrolysis for its metabolism (thus no negative feedback by ATP), resulting in increased uptake of fructose by the liver where it is exclusively metabolised by fructokinase (Teff et al., 2009). At the end of the metabolism of fructose some of the constituent carbons are converted to fatty acids that leads to the increased production of TG's and very low-density lipoprotein cholesterol (LDL-C) (Hannou et al., 2018). Simultaneously, fructose inhibits hepatic lipid oxidation, favouring synthesis of VLDL-triglycerides (Topping and Mayes, 1972). The lack of regulation of this pathway results in the excessive production of very-low density lipoprotein packed TGs in the liver (Tappy and Le, 2010). Part of the fructose taken up by the liver is also converted into fatty acids through the process of de novo lipogenesis, this may directly contribute to the development of NAFLD (Abdelmalek et al., 2010).

It is for this reason fructose is considered to be lipogenic, because unlike glucose, its conversion to glycerol-3-phosphate bypasses glycolysis which is a rate-limiting process. It is one of the reasons why consumption of a high fructose diet can result in synthesis of TGs from uncontrolled pathways (Kelishadi, Mansourian and Heidari-Beni, 2014). In addition, chronic fructose intake, unlike other sugars, does not stimulate a surge in leptin, a satiety indicating hormone (Miller and Adeli, 2008) which may be an additional mechanism for weight gain.

1.4 Treatment of metabolic syndrome and NAFLD

1.4.1 Conventional Treatments

Weight loss, dietary modifications and living a more physically active lifestyle have been proven to improve all of the components of MetS concurrently and are considered the first line of treatment (Kaur, 2014; Taylor, 2010). Several drugs are available to manage specific components of MetS. These include statins, fibrates, metformin and orlistat.

Statins are commonly used for the treatment of high cholesterol, as well as to manage and prevent, cardiovascular and coronary diseases (Hess et al., 2018; Parsaik et al., 2014). They are 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (Stancu and Sima, 2018). The beneficial effect of an HMG-CoA reductase inhibitor is a result of its similarity to endogenous HMG-CoA therefore this results in competitive, reversible inhibition of HMG-CoA reductase which is a rate determining step in cholesterol synthesis (Mcfarland et al., 2014). Unfavourable side effects of statin therapy include neurological and neurocognitive effects (Abramson et al., 2016), new-onset of T2D, hepatotoxicity and renal toxicity (Ward et al., 2019).

Fibrates are agents used to manage dyslipidaemia associated with MetS and T2D, particularly the elevated LDL-C and HDL-C (Jun et al., 2010). Fibrates reduce plasma levels of TGs by 30 % - 50 % and increase HDL-C levels by 5 % - 15 %, depending on baseline concentrations (Chapman, 2006). Fibrates increase the clearance of LDL particles (Chapman, 2003; Staels et al., 1998). Fibrate treatment results in the formation of LDL with a higher affinity for the LDL receptor, and are thus catabolized more rapidly (Caslake et al., 1993). Increased HDL production and stimulation of reverse cholesterol transport also occurs and thus increases plasma HDL-C concentrations (Berthou et al., 1996; Vu-Dac et al., 1995). Fibrates increase the production of HDL precursor proteins apolipoproteins A-I and A-II (apoA-I and apoA-II) in liver as a consequence this results in the elevation of HDL-C particles (Staels et al., 1998).

Metformin (1,1-dimethylbiguanide hydrochloride.) is a guanidine derivative, under the class biguanides (Taylor, 2010). It is the first line of treatment of T2D, particularly in obese individuals where diet is not adequately used to control diabetes (Nasri and

Rafieian-Kopaei, 2014). It acts as an insulin-sensitizing agent by reducing hepatic production and absorption of glucose in the small intestines and decreases fatty acid oxidation (Harrigan et al., 2001). Treatment with metformin decreases fasting plasma glucose concentrations by 20 % to 30 % in addition to decreasing glucose production (Fatima et al., 2018). Lactic acidosis which may have fatal consequences is a side effect associated with metformin treatment (Nathan et al., 2006). Metformin also results in gastrointestinal intolerance in which patients present with symptoms like diarrhoea, bloating, flatulence, nausea, and heartburn (Bailey, 2017; Vigneri and Goldfine, 1987).

Orlistat (tetrahydrolipstatin) is a semisynthetic hydrogenated derivative of *streptomyces toxytricini* (Al-Suwailem et al., 2006), that acts as a pancreatic and gastric lipase inhibitor and is used as an anti-obesity drug (Duvnjak et al., 2009). Orlistat reduces fat absorption by up to 30 % (Hauptman et al., 1992) and when used in conjunction with lifestyle modifications results in weight loss (5.8 kg vs 3.0 kg with placebo) and reduces progression of T2D (Khetan and Rajagopalan, 2018). Treatment with orlistat results in gastrointestinal side effects such as fatty stool, diarrhoea, flatulence and oily spotting (Padwal and Majumdar, 2007).

The adverse side effects of these conventional drugs have limited their use in many patients. Currently, there are no drugs developed to holistically treat the components of MetS and NAFLD simultaneously (Ebrahimof and Mirmiran, 2013). The use of herbal medicines (phytotherapy) is becoming widespread due to their holistic nature of therapy and their perceived lesser toxicity and less adverse reactions in comparison to synthetic agents (Verma and Singh, 2008).

1.4.2 Phytotherapy

Plants that possess phytochemicals with therapeutic potential are referred to as medicinal plants. Medicinal plants have been used in traditional medicine for thousands of years in African and Asian countries. The World Health Organisation (WHO) estimates that 60 % of the world population and about 80 % of developing countries are dependent on herbal medicines for basic healthcare needs (The World Health Organisation, 1999).

Biologically active chemical compounds found in plants are responsible for their healing properties (Minakshi et al., 2016). Phytochemicals can be found in variety of fruits, vegetables, legumes, teas, herbs and spices and they have been found to possess many therapeutic properties that are both protective and serve as treatment of human disease, if adequately consumed (Koche et al., 2018). Phytochemicals accumulate in different parts of the plant such as in the roots, leaves, steams, seeds, flowers and fruits (Saxena et al., 2013). Conventionally biologically active medicinal plant parts are prepared in various ways, either fresh or dried, usually by air drying the plant in the shade (Azwanida, 2015). The plant parts of interest can then be further crudely processed and consumed as powders, teas, infusions or decoctions for oral intake (Rashid et al., 2018; Wyk and Gorelik, 2017).

Crude extracts from medicinal plants tend to contain several phytochemical compounds, however for scientific validation it is important to consider each phytochemical individually (Yoo et al., 2018). Examples of biologically active phytochemicals include phenolic acids (gallic acid, chlorogenic acid), flavonoids (quercetin), organosulphurs (allicin) and curcuminoids (curcumin) (Alappat and Awad, 2010; Lee et al., 2013; Williams et al., 2013). Several of these phytochemicals have been identified as having anti-obesity and anti-inflammatory properties that protect against obesity and its related adverse health outcomes. It is stated in literature that phytochemicals target different stages in the life cycle of an adipocyte. This may be by reducing adipose tissue mass by inhibiting precursor cell proliferation and increasing apoptosis of adipocytes (Mukherjee et al., 2015; Yun, 2010). The phytochemical of interest for this study was the flavonoid quercetin.

1.4.3 Quercetin

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a dietary flavonoid, which exists widely in fruits and vegetables like, capers, black currants, onions, tomatoes and lettuce (D'Andrea, 2015). In plants, quercetin is usually bound to sugars, ethers or phenolic acids. Quercetin is a lipophilic compound with low water solubility and bioavailability, chemical instability and short biological half-life (Kasikci and Bagdatlioglu, 2016; Riva et al., 2019). Different forms of quercetin derivatives have different rates of absorption in the small intestine and stomach (Walle, 2004). Dietary quercetin is digested in the intestine and liver, and the metabolised and unmetabolised quercetin and its derivatives (3'-*O*-methylquercetin (isorhamnetin) and 4'-*O*-methylquercetin

(tamaraxetin) gradually accumulate in the liver, kidneys and other organs (Wang et al., 2016). Thus, habitual consumption of a quercetin-rich diet may affect the liver.

Quercetin has antioxidant properties (Kobori et al., 2009, 2011) and has been investigated in the management of diseases of the cardiovascular system and conditions associated with metabolic dysfunction. Experimental studies have shown that oral administration of 100 mg/kg of quercetin in streptozotocin diabetes induced rats, significantly reduced the elevation of plasma glucose (Kim et al., 2011) and according to Panchal, Poudyal and Brown (2012), animal models of hypertension and human participants given quercetin showed a significant decrease in systolic blood pressure. Quercetin intake also resulted in a reduction in body mass of obese Zucker rats whilst maintaining their average daily intake of feed (Rivera et al., 2008). The disease preventative properties of quercetin may be through acting on the expression of genes controlling the expression of Fatty Acid Synthase (FAS), Sterol Regulatory Element-Binding Protein (SREBP)-1, and by increasing Acetyl-CoA Carboxylase (ACC) phosphorylation (Portillo, 2011) and also scavenging of free radicals, inhibiting lipid peroxidation, and other anti-oxidative actions (Boots et al., 2008; Kobori et al., 2011).

1.5 Developmental programming

1.5.1 Developmental Origins of Health and Disease (DOHaD) hypothesis

The developmental origins of health and disease (DOHaD) hypothesis suggests that environmental conditions during developmentally plastic periods such as the foetal and early postnatal phases, impact health later on in life through permanent effects on growth, structure and metabolism (Barker, 2007; Bruce and Hanson, 2010; Reddy and Mbewu, 2016). Intrauterine and early postnatal life have been proposed as crucial windows where health outcomes in later life can be altered (Ekelund et al., 2007). The DOHaD hypothesis has emphasised the relationship between the peri-conceptual, foetal, and early phases of life and the manifestation of obesity and related metabolic disorders in later life (Vickers, 2014) or improved health through the programming of genes and/or altered gene expression (Reddy and Mbewu, 2016). Previous studies have mainly focused on maternal over-nutrition, high fat consumption during pregnancy or foetal under nutrition and the resultant development of outcomes associated with MetS and renal disease in adulthood

(Jackson et al., 2012). Relatively few studies have investigated the early post-weaning period as a target for interventions in developmental programming.

1.5.2 The early post weaning period as a target for developmental programming

The early post-weaning period, when animals make the transition from a milk only diet to solid food, is associated with hormonal, enzyme and metabolic modifications in the individual. Hormonal changes during the weaning period include an increase in plasma insulin concentrations in rats weaned onto a high fat diet (Blazquez et al., 1970). Enzyme modifications occurring during weaning include alterations in both digestive and metabolic enzymes. There is a reduction in lactase and lysosomal neuraminidase expression that is high during suckling but whose expression is reduced after weaning (Neal-Kluever et al., 2019). During weaning there is a notable increase in the expression of α-amylase and chymotrypsin, lipase and trypsin in the small intestines and to a smaller extent in the pancreas as well (Robberecht et al., 1971). There are also significant morphological changes that occur in the pancreas. For example, during weaning the size of the pancreas increases as rats transition to a solid diet (Anzi et al., 2018).

Regarding metabolic enzyme changes at weaning, a significantly increased (between six-to ten-fold) enzyme activity of fatty acid synthase (FAS), malic enzyme (ME) and citrate cleaved enzyme (CCE) was observed in the livers of rat pups, for the first five days after weaning (Hahn and Novak, 1975; Taylor et al., 1967). Other experimental studies have also supported the observation of a significant increase in FAS and acetyl-Co carboxylase mRNA levels and in the activity of lipogenic enzymes that occur in white adipose tissue and the liver of rats weaned onto a high-carbohydrate diet (Girard et al., 1994). Surges in the expression and activity of lipogenic enzymes during this developmental period may increase later disease risk (Conceição et al., 2015).

It was also reported that 6-phosphogluconate dehydrogenase (6-PGDH) and glucose-6-phosphate dehydrogenase (G6PDH) also increased by 30 % at weaning from their steady pre-weaning levels (Bazin and Lavau, 1982). The increase in these enzymes plays an important role in carbohydrate metabolism.

Recent evidence has also shown that there are significant changes in the gut microbiome which occur at weaning, due to the role played by diet change in influencing gut microbiota, regardless of genetic influence (Carmody et al., 2011; Flemer et al., 2017). It has been noted that during the weaning period there is a reduction in the *Lactobacillaceae, Bifidobacteriaceae, Enterococcaceae* and Enterobacteriaceae and an increase in *Lachnospiraceae, Ruminococcaceae* and *Bacteroidaceae* species due to the transition from a milk only diet to the introduction of solid food (Laursen et al., 2017). Deterrents to proper bacterial colonisation in early life have been hypothesised to result in autoimmune disorders (Voreades et al., 2014). Later in life, the intestinal microbiome has been associated with weight regulation through its role in dietary energy extraction, regulation of fat storage and appetite control (Tilg and Kaser, 2011). In this regard, obese individuals have been reported to have an abundance of *Firmicutes* and nearly 90 % less *Bacteroidetes* than lean individuals (Ley et al., 2005) and upon changing to a low caloric diet it was observed that there was an increase in the *Bacteroidetes* bacterial community and reduction in *Firmicutes* (Davis, 2016).

1.6 Rationale for study

Given the increasing prevalence of metabolic disorders which place a heavy burden on healthcare facilities, there is a need to develop innovative approaches for managing diseases. The current therapies tend to be mono-therapeutic and require chronic use by patients which adds to the cost of management and reduces patient compliance. Therefore, naturally occurring phytochemicals, like quercetin, that possess antioxidant and anti-obesogenic properties, should be considered as possible interventions used to manage or prevent metabolic disorders. Strategic interventions during periods of developmental plasticity could have long lasting positive impacts on the metabolic health of individuals. For example, Nyakudya et al., (2018) found that administration of oleanolic acid in the neonatal phase prevented the development of high fructose diet induced NAFLD. Lembede et al., (2018) reported on the effects of neonatal orally administered S-allyl cysteine in high-fructose diet fed Wistar rats and found that S-allyl cysteine prevented the development of fructose-induced liver lipid accumulation in adulthood. Ibrahim et al., (2019) investigated the long-term protective effects of neonatal administration of

curcumin against non-alcoholic steatohepatitis in high-fructose-fed adolescent rats where it was found that curcumin administration protected the rats against the development of fructose induced non-alcoholic steatohepatitis by causing a downregulation of hepatic gene expression of alpha-adenosine monophosphate and an upregulation of Tumour Necrosis Factor alpha in both male and female rats.

As discussed earlier the early post-weaning period is characterised by profound, hormonal, enzyme, structural, microbiome, functional and metabolic changes. It is during this period that lipogenic enzymes, for example acetyl-Co-A-carboxylase, malic enzyme (decarboxylating) and glucose-6-P dehydrogenase appear for the first time in the liver and adipose tissue. Therefore, it seems logical that by supressing the early increase in the expression and activity of lipogenic enzymes through strategic dietary interventions with biologically active phytochemicals, the lipogenic capacity of tissues and organs when exposed to lipogenic diets, such as a high fructose diet, could be attenuated, thus preventing the development of metabolic dysfunction (e.g. NAFLD and obesity). Previous studies have a heavy bias for males (Beery, 2018). This is despite previously reported sexual dimorphic responses to metabolic challenges (Klein et al., 2015). There is therefore need to consider both sexes in such studies.

1.7 Aim

To investigate whether the flavonoid quercetin, when administered in the early post weaning period will prevent the development of negative health outcomes associated with high dietary fructose in male and female Sprague Dawley rats.

1.8 Objectives

To determine the effects of dietary quercetin supplementation during the early post-weaning period in Sprague Dawley rats fed high fructose diets, by determining:

- The growth performance of the rats, by assessment of body mass gain and linear growth as determined by the length, mass and relative density of the tibia and femur.

- The development of metabolic dysfunction through the assessment of general impairment of metabolism by determining:
  - circulating levels of metabolites (glucose, triglycerides and cholesterol),
  - adiposity (visceral and epididymal fat pad), and
  - the concentrations of key hormones (insulin and adiponectin) involved in regulation of metabolism.
- The development of metabolic dysfunction through the assessment of the liver for non-alcoholic fatty liver disease, specifically considering:
  - the development of fatty liver by histological scores of hepatic steatosis.
- The general health profile of the rats through the assessment of:
  - markers of liver function [alanine transaminase (ALT), total bilirubin and albumin].
  - renal function markers (creatinine and blood urea nitrogen).

1.9 Hypotheses

H_0: Quercetin administration (in the early post weaning phase) to Sprague Dawley rats fed a high fructose diet will not affect growth performance, nor will it prevent the development of metabolic dysfunction and related adverse health effects.

H_1: Quercetin administration (in the early post weaning) to Sprague Dawley rats fed a high fructose diet will affect growth performance, and also prevent the development of metabolic dysfunction and related adverse health effects.

CHAPTER 2: MATERIALS AND METHODS

2.1 Ethical approval of the study

The study was approved by the animal ethics screening committee of the University of Witwatersrand (AESC clearance certificate no. 2017/02/07B), with my supervisor (Dr J. Donaldson) as the principal investigator. I was subsequently included as a co-worker prior to data collection. A copy of the documentation is included in this dissertation as appendix A.

2.2 Animals and housing

2.2.1 Animals

Sixty-four (n= 64), 21-day old Sprague Dawley rat pups were used in the study (32 males and 32 females). The rats were sourced from the Central Animal Services unit of the University of Witwatersrand, Johannesburg (where study was undertaken in the same building) and were acclimatised to handling and housing for one day. The rats were randomly allocated to one of four treatment groups, with n= 16 rats in each group (8 males and 8 females).

2.2.2 Housing and general care

The rats were housed individually in Perspex cages containing wood shavings for bedding, in the Central Animal Service unit of the University of Witwatersrand, Faculty of Health Sciences. Bedding was replaced once a week. A standard twelve-hour light: twelve-hour dark cycle (lights switched on between 7h00 and 19h00) and room temperature of 24 ± 2 ºC were maintained during the study period.

2.2.3 Study design

The study was divided into two major phases. In the first phase which extended from when the rats were 21 days old until they were 35-days old, the Sprague Dawley rat pups were randomly allocated to one of four treatment groups replicated by sex. Group1 (negative control) received standard rat chow (SRC), tap water to drink *ad libitum* and plain gelatine cubes, twice per day. Group 2, in which the effects of quercetin alone were investigated, received SRC, tap water to drink *ad libitum* and gelatine cubes containing quercetin (100 mg/kg body mass) (Kim et al., 2011; Chung et al., 2013), twice per day. Group 3 received SRC and 20 % fructose drinking

solution as drinking (Meirelles et al., 2011) fluid *ad libitum* to induce metabolic dysfunction and plain gelatine cubes, twice per day. Group 4 received SRC, 20 % fructose drinking solution *ad libitum* as drinking fluid and gelatine cubes containing quercetin (100 mg/kg body mass), twice daily in order to investigate whether quercetin intake in the early post-weaning period protects against diet induced metabolic dysfunction. The gelatine cubes, with and without quercetin, were placed into the rat cages on a white piece of paper and the rats which were housed individually, were observed until they ate the cubes.

From post-natal day 36, the rats continued to the second phase of the study where they continued to receive SRC and either normal tap water or 20 % fructose drinking solution as drinking fluid, as per group allocation, but without gelatine cubes and their associated treatments. The second phase of the study lasted for 42 days. Figure 2.1 shows a summary of the group allocations and treatment regimens followed during the two experimental phases.

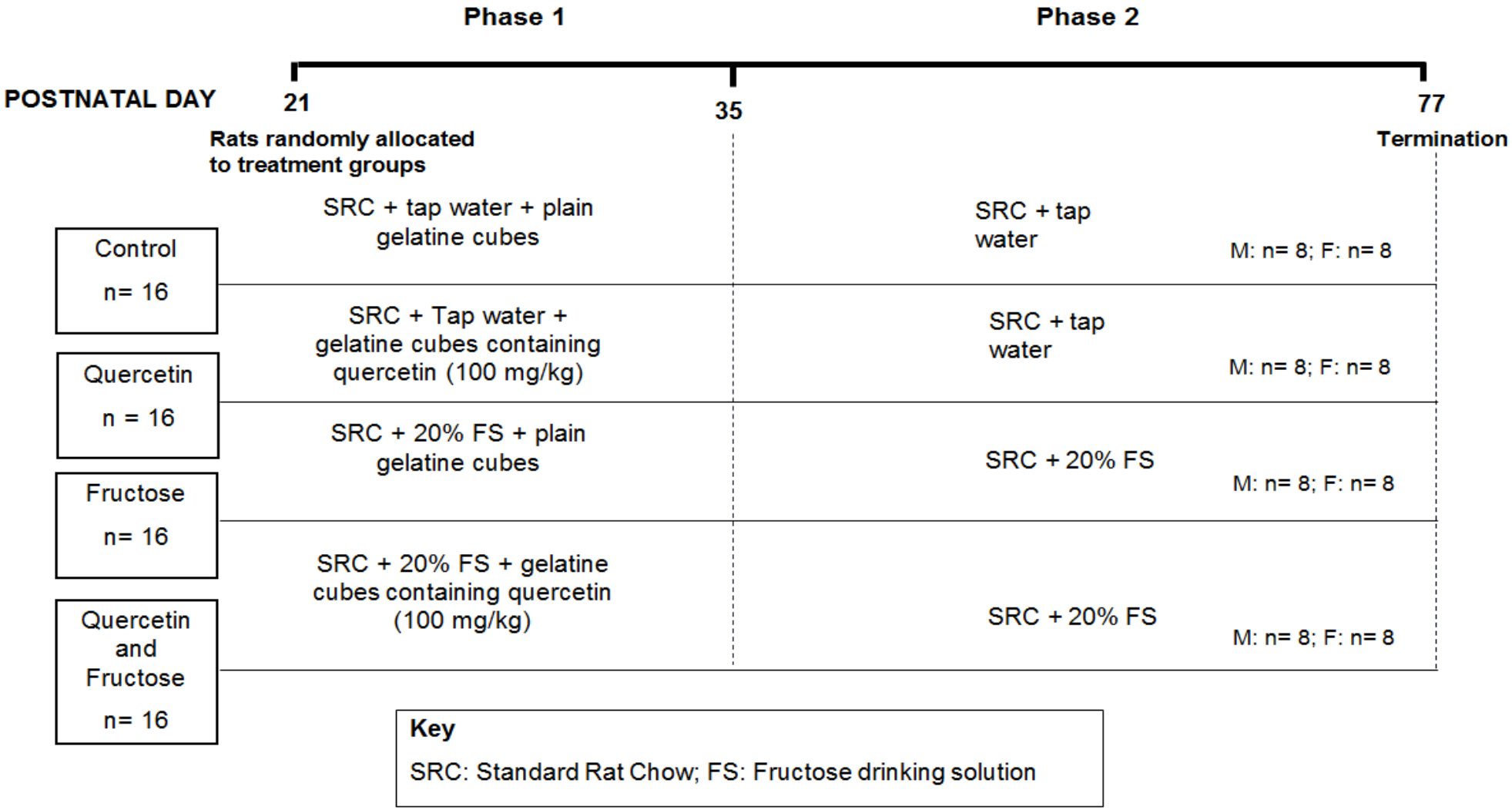

Figure 2.1: Group allocations, timeline and treatment regimens

2.3 Gelatine cube and fructose drinking solution preparation

Gelatine cubes were prepared by combining gelatine (Sheridans gelatine, Libstar Operations (Pty) Ltd, Dunkeld, South Africa), 8 g brown sugar (Brown sugar, Selati, RCL Foods Ltd,, Westville, South Africa) and 3 ml bovril (Beefy Bovril, Pioneer Foods (Pty) Ltd, South Africa), into 100 ml of hot water. Quercetin powder (Quercetin, Sigma-Aldrich Co., St Louis, United States of America) (dose adjusted to rat body mass) was mixed with 0.5 % dimethyl sulfoxide (DMSO) (Dimethyl sulfoxide, Sigma-Aldrich Co., St Louis, United States of America) as a solvent before adding it to the gelatine mixture. The mixture was then poured into 2 ml moulds and allowed to set in a refrigerator at 4°C.

The twenty percent (20 %) fructose drinking solution was prepared by adding 400 g fructose powder (Fructose, Nature's Choice, Randvaal, South Africa) to 2000 ml of hot water and two drops of red food colouring (Robertson Food Colouring, Libstar Operations (Pty) Ltd, Dunkeld, South Africa). This was then mixed until fully dissolved. Water bottles were changed bi-weekly. Two drops of green food colouring (Robertson Food Colouring, Libstar Operations (Pty) Ltd, Dunkeld, South Africa) were also added to the tap water provided to the other groups in order to distinguish it from the red coloured fructose drinking solution.

2.4 Body mass measurements

An electronic balance (Snowrex EQ-1200, Snowrex International Company, Taipei, Taiwan) was used to weigh the rats daily during the first two weeks to monitor growth performance and adjust the amount of the quercetin to ensure a consistent dosage relative to body mass. Thereafter the frequency of weighing was reduced to twice a week.

2.5 Terminal procedures

Following the second phase of the study, the rats were fasted overnight, where only tap water was provided. Following a pin prick to the tail, blood was collected, and fasting glucose was measured using a calibrated glucometer (Contour plus Blood glucose monitoring system, Bayer (Pty) Ltd., Praha, Czech Republic). Haemoglobin

and haematocrit levels were also measured using an HCT meter (InSight HCT Meter, Woodley Equipment Company Ltd, Bolton, England). The rats were then euthanized using a sodium pentobarbital overdose (Euthapent, Kyron laboratories, Johannesburg, South Africa) at 200 mg/kg body mass injected intraperitoneally. Tissue and blood samples were then collected for further analysis.

2.6 Blood parameters

Blood samples were collected by cardiac puncture into vacutainer coagulant coated tubes (Vacuette tube, Greiner Bio-One, Kremsmünster, Austria). The blood was then centrifuged (at 1500 g for 20 minutes) in order to obtain serum for the clinical biochemistry (TGs, albumin, ALT, total bilirubin, creatinine, BUN and HDL and LDL cholesterol) and hormone measurements (insulin and adiponectin). The serum was then stored at - 20 ºC until assays were performed.

2.6.1 Clinical biochemistry assay

Serum TGs and surrogate markers of liver (alanine transaminase (ALT) and total bilirubin) and renal function (creatinine and blood urea nitrogen) were measured using an IDEXX Chemistry Analyser (IDEXX VetTest® Clinical Chemistry Analyzer, IDEXX Laboratories Inc., USA) according to manufacturer's instructions.

2.6.2 Insulin and adiponectin concentrations

Fasting insulin and adiponectin levels were determined using ELISAs (Elabscience Biotechnology Co., Ltd, Wuhan, China). Kit reagents and serum samples were brought to room temperature. Concentrated wash buffers (25x) were diluted to a 1x working wash buffer. Different concentrations (20, 10, 5, 2.5, 1.25, 0.63, 0.31 ng/mL) of the insulin reference standard was prepared as well as for the adiponectin reference standard (3000, 1500, 750, 375, 187.5, 93.75, 46.88 pg/mL) in order to generate a standard curve which was then used to determine the insulin and adiponectin concentrations in the samples. Biotinylated Detection Ab and Concentrated HRP conjugate were diluted from 100x to 1x working solution. 100 µL of standards and serum samples were added to each microplate well and incubated at 37°C for 90 minutes. Assay procedure for Sandwich-ELISA was then followed according to manufacturer's instructions. Optical density (OD) value of each well was determined at 450nm using a Labsystems Multiskan Ascent plate reader and results

were calculated. To calculate duplicate readings for each standard and sample were averaged, and then averaged zero standard OD was subtracted from readings. GraphPad prism version 5 software (Graph-pad Software Inc., San Diego, USA.) was used to log transform the row data and determine serum concentrations.

2.6.3 HDL/ LDL cholesterol

Serum HDL and LDL levels were determined using an ELISA kit (HDL and LDL/VLDL Cholesterol Assay kit, Cell Biolabs, Inc., San Diego, United States of America). Serum and assay diluent samples were thawed and brought to room temperature. Assay diluent and cholesterol reaction reagents were diluted as per manufacturer's instructions. Precipitation reagent (200 µL) was added to 200 µL serum samples in microcentrifuge tubes, vortexed and incubated for 10 minutes at room temperature to allow precipitation to occur. Thereafter, the samples were centrifuged and the supernatant (the HDL fraction) was transferred into new microcentrifuge tubes. The pellet (the LDL fraction) was dissolved and re-suspended using PBS. Further dilutions of both fractions were required in order to run the assay. After further dilution of both HDL and LDL samples, 50 µL of the standards and samples and 50 µL cholesterol reaction reagents were pipetted into a microtiter plate and covered to protect the reaction from light. The plate was then incubated for 45 minutes at 37°C. Following incubation, the plate was read with a fluorescent microplate reader (Cytation 5 Image Multi-Mode reader, Bio Tek Instruments, Vermont, United States of America), equipped for excitation in the 530-570 nm range and for emissions in the 590-600 nm range.

2.7 Tissue Parameters

2.7.1 Body adiposity

Visceral and epididymal fat (male rats) deposition was quantified by dissecting out and then weighing the absolute mass of the mesenteric, retroperitoneal and epididymal (males only) fat pads using a balance (Precisa 310 M, Precisa Gravimetrics AG, Dietikon, Switzerland). The relative (to body mass) mass of these fat depots was then computed.

2.7.2 Liver histology

Once the rats were euthanized, the liver was excised and weighed. The left lobe of the liver was dissected and preserved in 10 % phosphate buffered formalin (PBF). After fixation in the PBF, a piece of the liver was cut and processed for histology using an automated tissue processor (Citadel 2000, Thermo Fisher Scientific, Runcorn, United Kingdom). In summary, the liver tissue sample was transferred to a tissue cassette and dehydrated through graded and absolute alcohols for approximately ten and a half hours. The tissue was then cleared in chloroform twice for three hours and infiltration of paraffin wax occurred for three and a half hours. The liver samples were then embedded in paraffin wax and allowed to set before the blocks were sectioned using a microtome (Leica Biosystems Instruments (Pty), Ltd, model RM 2125, Wetzlar, Germany). Sections were subsequently mounted onto glass slides. Haematoxylin and eosin stains were used for histological examination using a light compound microscope (Axioskop 2 plus microscope, Carl Zeiss (Pty) Ltd, Göttingen, Germany) provided with CCD camera (AxioCam HRc, Carl Zeiss (Pty) Ltd, Göttingen, Germany) and image capture software (ZEN 2011, Carl Zeiss (Pty) Ltd, Göttingen, Germany). The liver slides were scored for the presence of micro- and macrosteatosis as described by Brunt et al., 2011, see table 2.1 below.

Table 2.1: Micro- and Macro-steatosis score cut off values (Brunt et al., 2011). Hepatocellular hypertrophy was semi-quantitatively analysed by determining the percentage area affected in each camera field.

Steatosis grade	**Grade 0**	**Grade 1**	**Grade 2**	**Grade 3**
Microsteatosis	< 5 %	5–33 %	34–66 %	>66 %
Macrosteatosis	< 5 %	5–33 %	34–66 %	>66 %

2.7.3 Linear growth

The left femora and tibiae were disarticulated, defleshed, dried in an oven and then weighed. Vernier callipers (KTV150, 150mm Digital Caliper, Major Tech (Pty) Ltd, Elandsfontein, South Africa) were then used to measure the bone lengths. Bone density was then estimated using the formula:

Bone density = mass of bone (mg)/ length of bone (mm).

2.8 Data Analysis

The parametric data are expressed as mean ± standard deviation. Non-parametric liver steatosis scores are expressed as median (interquartile range). GraphPad prism version 5 software (Graph-pad Software Inc., San Diego, USA.) was used for data analysis and graph plotting. Dietary groups were compared using a one-way ANOVA for both male and female rats, separately. This was followed by a Tukey's post hoc test for comparison of the means. Differences were considered statistically significant if $P \leq 0.05$. The Kruskal–Wallis test (non- parametric one-way ANOVA) was used to analyse NAFLD steatosis scores of multiple group data followed by Dunn's post hoc test to compare the medians.

CHAPTER 3: RESULTS

3.1 Mortality and morbidity

All animals remained apparently healthy during the study period, with no record of mortality or morbidity.

3.2 Effects of orally administered quercetin on growth performance

3.2.1 Body mass

3.2.1.1 Male rats

Figure 3.1 shows the effects of orally administered quercetin on the body mass of male Sprague Dawley rats fed a high fructose diet for eight weeks. There were no significant differences ($p > 0.05$) across treatment groups in the initial body mass of the rats, nor in the body mass at the end of the first phase of the study (day 35). All rats grew significantly during the first and second phase of the study ($p < 0.0001$). However, at the end of the second phase of the study period, after 8 weeks, the rats receiving fructose and those receiving fructose + quercetin had significantly lower final body masses than the rats receiving only quercetin ($p <0.05$).

Figure 3.2 shows the effects of orally administered quercetin on the percentage body mass gain of male Sprague Dawley rats fed a high fructose diet for eight weeks. Panel A shows the percentage body mass gain during the first study phase (Postnatal day 21–35) where rats receiving fructose and those receiving fructose + quercetin had a significantly lower ($p < 0.05$) percentage body mass gain compared to those receiving quercetin only. Percentage body mass gain was not significantly different ($p > 0.05$) between treatment groups during the second phase Panel B (postnatal day 35–77) or after the entire eight week feeding period (Panel C).

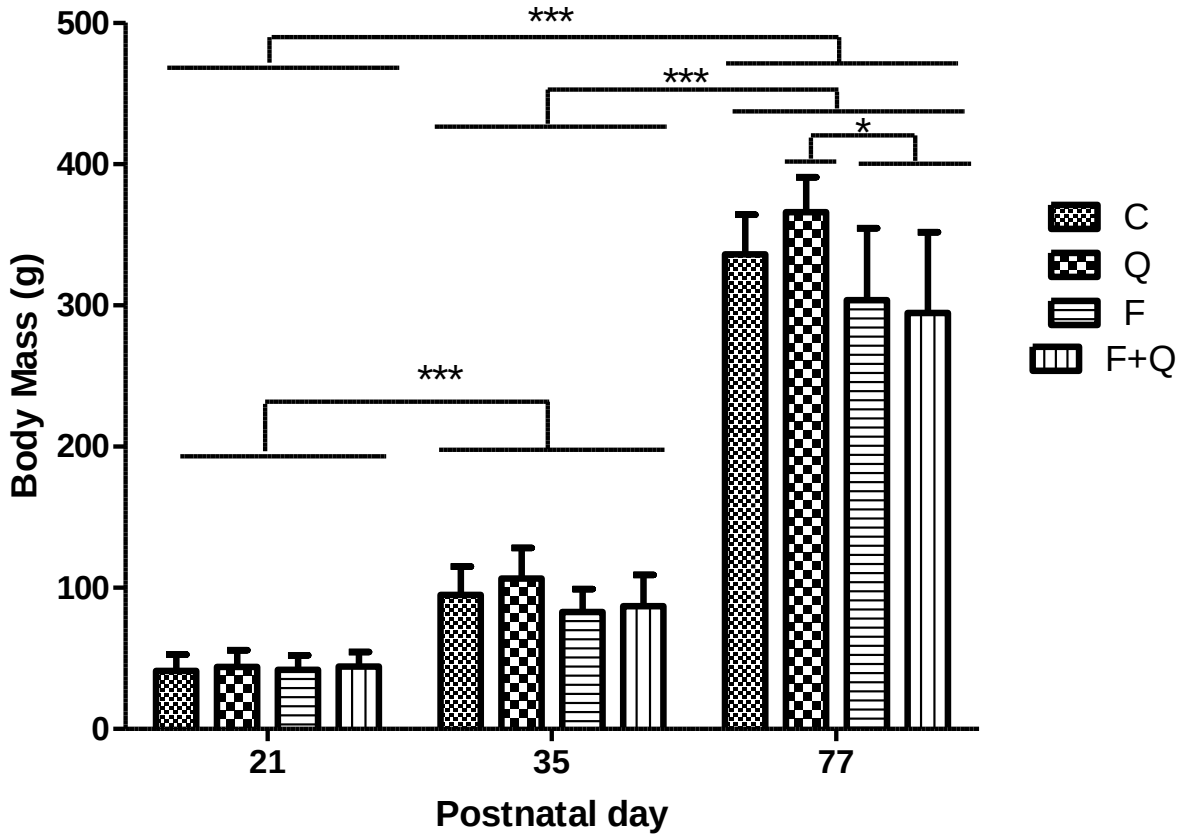

Figure 3.1: The effects of orally administered quercetin on the body mass of male Sprague Dawley rats fed a high fructose diet for eight weeks. Initial mass (postnatal day 21), body mass following the first phase - at postnatal day 35 and terminal body mass (postnatal day 77). C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8. Data represented as mean ± standard deviation; *** $p < 0.0001$; * $p < 0.05$.

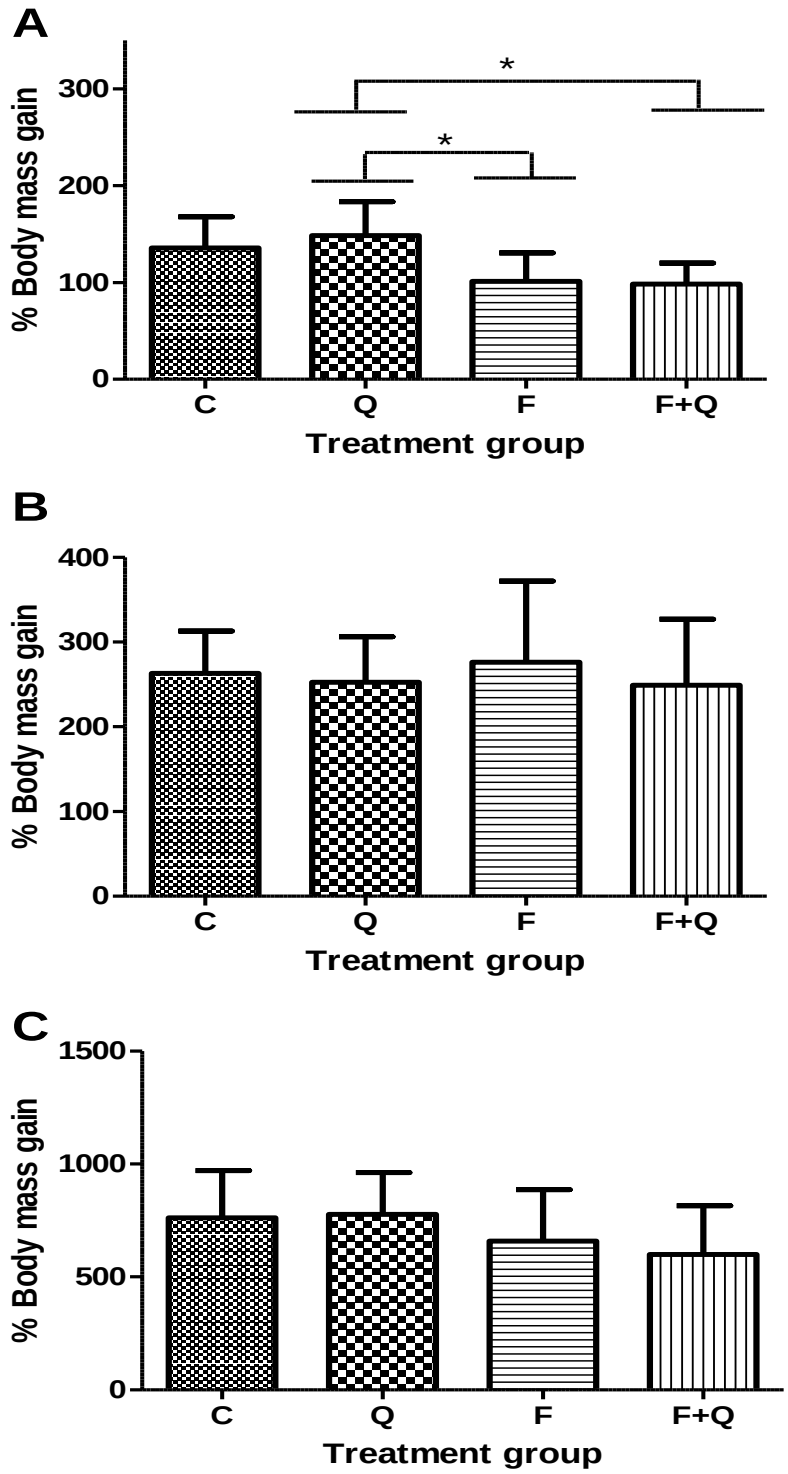

Figure 3.2: The effects of orally administered quercetin on the percentage body mass gain of male Sprague Dawley rats fed a high fructose diet for eight weeks. Panel A (Postnatal day 21 – 35), Panel B (Postnatal day 35 – 77) and Panel C (Postnatal day 21 – 77). C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8. Data represented as mean ± standard deviation; * $p < 0.05$.

3.2.1.2 Female rats

Figure 3.3 shows the effects of orally administered quercetin on the body mass of female Sprague Dawley rats fed a high fructose diet for eight weeks. There were no significant differences ($p > 0.05$) across treatment groups in the initial body mass of the rats, nor in the body mass at the end of the first phase of the study (day 35). All rats grew significantly during both the first (postnatal day 21 – 35) and second phase (postnatal day 35 – 77) of the study ($p < 0.0001$). No significant differences in final body mass were observed across the treatment groups ($p > 0.05$).

Figure 3.4 shows the effects of orally administered quercetin on the percentage body mass gain of female Sprague Dawley rats fed a high fructose diet for eight weeks. Panel A shows the percentage body mass gain of rats during the first phase of the study (postnatal day 21 – 35) where rats receiving fructose ($p < 0.05$) and those receiving fructose + quercetin ($p < 0.01$) had significantly lower percentage body mass gain compared to control rats and those receiving quercetin only. Panel B shows the percentage body mass gain of rats during the second phase of the study (postnatal day 35 – 77), where rats receiving fructose and those receiving fructose + quercetin ($p < 0.05$) had a significantly higher percentage body mass gain compared to control rats. No significant differences ($p > 0.05$) in percentage weight gain across treatment groups, over the entire feeding period, were observed (Panel C).

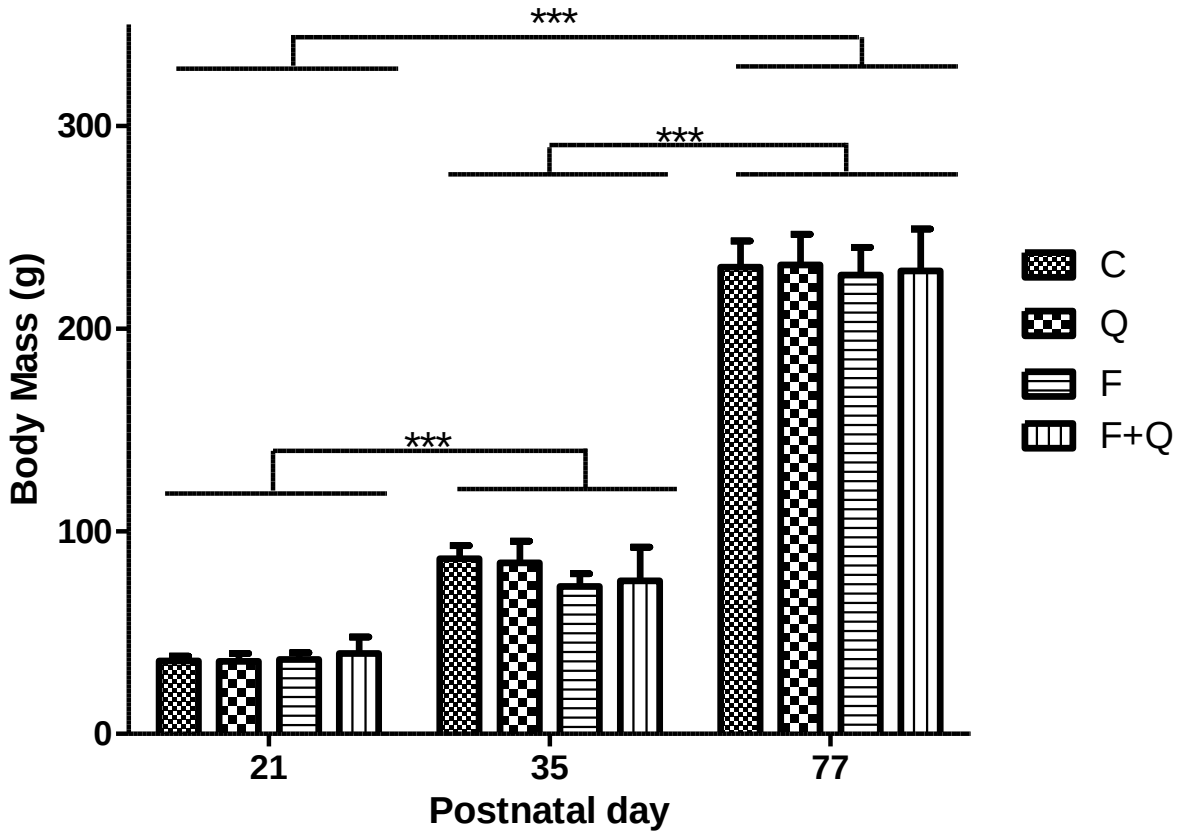

Figure 3.3: The effects of orally administered quercetin on the body mass of female Sprague Dawley rats fed a high fructose diet for eight weeks. Initial mass (postnatal day 21), body mass at postnatal day 35 and terminal mass (postnatal day 77). C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8. Data represented as mean ± standard deviation; *** $p < 0.001$.

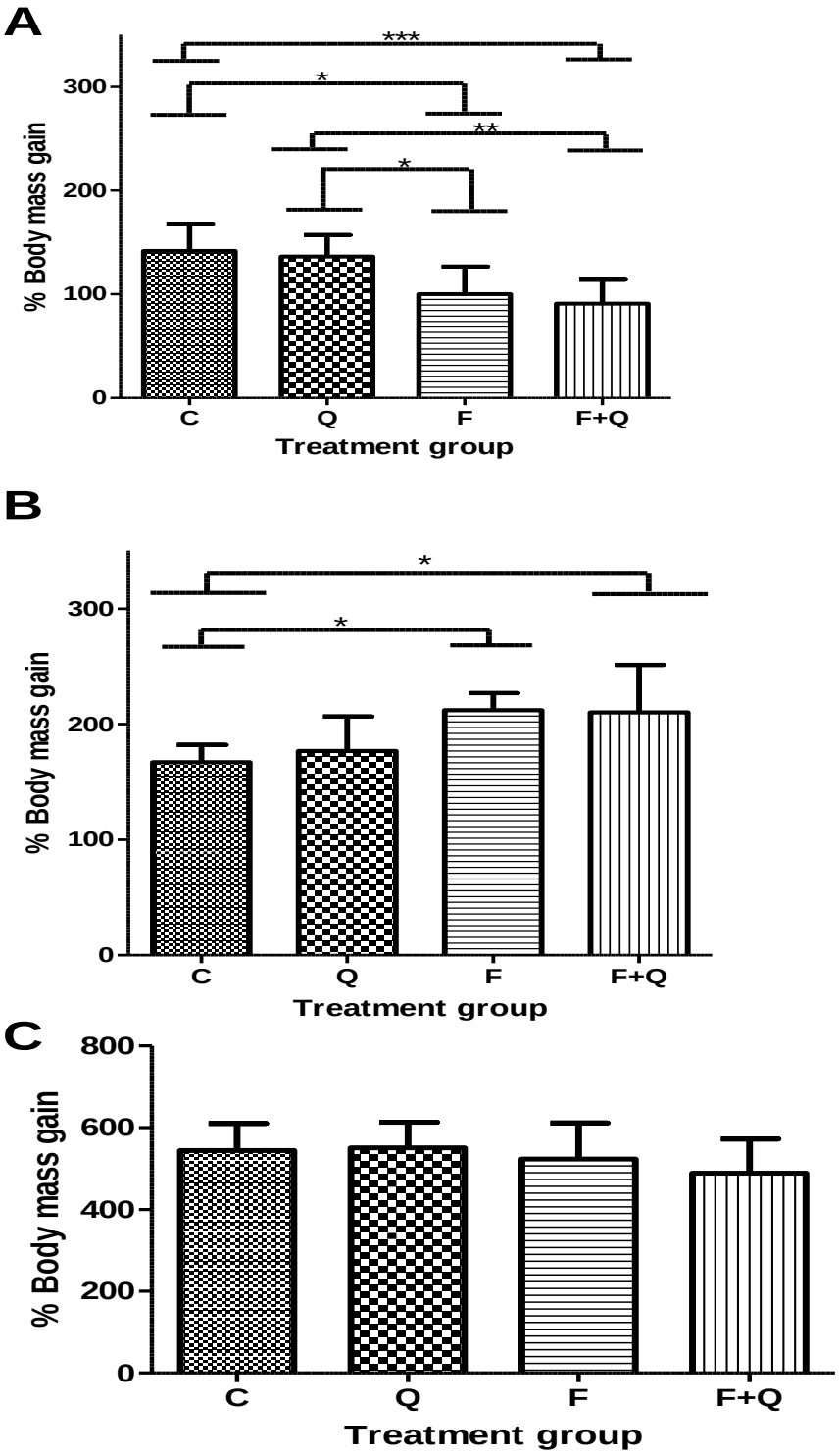

Figure 3.4: The effects of orally administered quercetin on the percentage body mass gain of female Sprague Dawley rats fed a high fructose diet for eight weeks. Panel A (Postnatal day 21 – 35), Panel B (Postnatal day 35 – 77) and Panel C (Postnatal day 21 – 77). C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8. Data represented as mean ± standard deviation, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

3.2.2 Linear growth

3.2.2.1 Male rats

Table 3.1 shows the effects of orally administered quercetin on the tibia and femur bone mass, length and mass to length ratio of male Sprague Dawley rats fed a high fructose diet for eight weeks. Rats receiving fructose had significantly lighter and shorter tibias than control rats ($p < 0.05$) and rats receiving quercetin alone ($p < 0.01$). Rats receiving fructose + quercetin ($p < 0.05$) had significantly lighter and shorter tibias than those receiving quercetin alone. There were significant differences in the tibial mass to length ratio of rats in the fructose group having lower tibial mass to length ratio compared to control group ($p < 0.05$) and quercetin group ($p < 0.01$) rats.

The femora of rats receiving fructose and rats receiving fructose + quercetin were significantly lighter and shorter than those of control rats ($p < 0.01$) and rats receiving quercetin alone ($p < 0.01$). Rats in the fructose ($p < 0.05$) and fructose + quercetin ($p < 0.05$) group had a significantly lower mass to length femoral bone ratio than those of the control group and quercetin group rats.

Table 3.1: The effects of orally administered quercetin on the tibia and femur bone mass, length and mass to length ratio of male Sprague Dawley rats fed a high fructose diet for eight weeks.

Parameter	C	Q	F	F + Q
Tibia mass (mg)	450.30 ± 37.78^{c}	469.50 ± 32.97^{ac}	371.20 ± 44.56^{b}	398.80 ± 48.61^{bc}
Tibia length (mm)	38.63 ± 0.64^{c}	39.16 ± 0.74^{ac}	36.62 ± 1.07^{b}	37.25 ± 1.42^{bc}
Tibia mass to length ratio	11.65 ± 0.74^{a}	11.99 ± 0.74^{a}	10.12 ± 1.02^{b}	10.68 ± 0.98^{ab}
Femur mass (mg)	570.30 ± 32.43^{a}	572.50 ± 43.96^{a}	467.30 ± 46.02^{b}	469.70 ± 57.21^{b}
Femur length (mm)	33.82 ± 1.08^{a}	33.95 ± 0.86^{a}	31.72 ± 0.75^{b}	31.83 ± 1.27^{b}
Femur mass to length ratio	16.86 ± 0.64^{a}	16.85 ± 1.07^{a}	14.71 ± 1.12^{b}	14.72 ± 1.27^{b}

Data represented as mean ± standard deviation. C = Control group - tap water and plain gelatine cubes, n = 6; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 6; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 6; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 6. Values in a row with different superscripts are significantly different ($p<0.05$).

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3.2.2.2 Female rats

Table 3.2 shows the effects of orally administered quercetin on the tibia and femur bone mass, length and mass to length ratio of female Sprague Dawley rats fed a high fructose diet for eight weeks. No significant differences ($p > 0.05$) in tibia mass were observed across treatment groups. Significant differences were also not observed in the length of the tibial bone in female rats with the exception of rats in the fructose + quercetin group having significantly shorter tibia than the control rats ($p < 0.05$). There were no significant differences in the tibia mass to length ratio across treatment groups.

There were no significant differences observed in the femora mass and mass to length ratio across treatment groups. Rats of the fructose + quercetin group had significantly shorter femora than that of control group ($p< 0.01$) and quercetin only ($p < 0.01$) rats. Rats of the fructose group also had significantly shorter femora than that of quercetin only rats ($p < 0.05$).

Table 3.2: The effects of orally administered quercetin on the tibia and femur bone mass, length and mass to length ratio of female Sprague Dawley rats fed a high fructose diet for eight weeks.

Parameter	C	Q	F	F + Q
Tibia mass (mg)	384.30 ± 33.05[a]	379.00 ± 14.97[a]	359.90 ± 36.56[a]	347.30 ± 27.42[a]
Tibia length (mm)	36.25 ± 1.06[a]	36.18 ± 0.65[ab]	35.53 ± 0.84[ab]	35.06 ± 0.55[b]
Tibia mass to length ratio	10.59 ± 0.67[a]	10.48 ± 0.36 [a]	10.11 ± 0.82 [a]	9.90 ± 0.67 [a]
Femur mass (mg)	485.90 ± 38.85[a]	489.90 ± 15.30 [a]	459.90 ± 49.40 [a]	457.0 ± 36.07 [a]
Femur length (mm)	31.43 ± 0.59[ac]	31.59± 0.49[a]	30.61 ± 0.67[bc]	30.33 ± 0.57[b]
Femur mass to length ratio	15.45 ± 1.05[a]	15.51 ± 0.48[a]	15.00 ± 1.32[a]	15.06 ± 0.98[a]

Data represented as mean ± standard deviation. C = Control group - tap water and plain gelatine cubes, n = 7; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 7; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 7; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 7. Values in a row with different superscripts are significantly different ($p<0.05$).

3.3 Effect of orally administered quercetin on circulating metabolites

3.3.1 Male rats

Table 3.3 shows the effects of orally administered quercetin on circulating metabolites of male Sprague Dawley rats fed a high fructose diet for eight weeks. No significant differences in blood glucose, serum TGs, HDL cholesterol and LDL cholesterol were observed across all treatment groups ($p > 0.05$).

Table 3.3: The effects of orally administered quercetin on circulating metabolites of male Sprague Dawley rats fed a high fructose diet for eight weeks.

Parameter	C	Q	F	F + Q
Glucose (mmol/L)	3.58 ± 0.36	3.63 ± 0.35	3.78 ± 0.47	3.59 ± 0.29
Triglycerides (mmol/L)	0.43 ± 0.15	0.39 ± 0.15	0.61 ± 0.22	0.53± 0.18
HDL (µM)	6.50 ± 0.58	6.05 ± 0.59	6.17 ± 0.48	7.32 ± 1.82
LDL (µM)	1.67 ± 0.23	1.70 ± 0.13	1.86 ± 0.20	2.16 ± 0.31

Data represented as mean ± standard deviation. C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8. HDL = High density lipoprotein cholesterol, LDL = Low density lipoprotein cholesterol.

3.3.2 Female rats

Table 3.4 shows the effects of orally administered quercetin on circulating metabolites of female Sprague Dawley rats fed a high fructose diet for eight weeks. No significant differences in blood glucose, serum HDL cholesterol and LDL cholesterol were observed across treatment groups ($p > 0.05$). Rats in the quercetin group had significantly lower serum TG levels than those in the fructose group ($p < 0.01$).

Table 3.4: The effects of orally administered quercetin on circulating metabolites of female Sprague Dawley rats fed a high fructose diet for eight weeks**.**

Parameter	C	Q	F	F + Q
Glucose (mmol/L)	3.60 ± 0.23	3.78 ± 0.18	3.68 ± 0.24	3.52 ± 0.52
Triglycerides (mmol/L)	0.45 ± 0.19 ^{ab}	0.34 ± 0.15^{a}	0.74 ± 0.30 ^{b}	0.65 ± 0.24 ^{ab}
HDL (µM)	9.18 ± 1.58	9.76 ± 1.55	9.27 ± 2.89	12.00± 1.052
LDL (µM)	1.83 ± 0.47	1.84 ± 0.46	2.73 ± 0.85	3.07 ± 1.07

Data represented as mean ± standard deviation. Values in a row with different superscripts are significantly different ($p < 0.05$). C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8. HDL = High density lipoprotein cholesterol, LDL = Low density lipoprotein cholesterol.

3.4 Effect of orally administered quercetin on adiposity

3.4.1 Male rats

Table 3.5 shows the effects of orally administered quercetin on the adiposity of male Sprague Dawley rats fed a high fructose diet for eight weeks. No significant differences in the absolute and relative (to body mass) visceral and epididymal fat mass were observed between treatment groups ($p > 0.05$).

Table 3.5: The effects of orally administered quercetin on the adiposity of male Sprague Dawley rats fed a high fructose diet for eight weeks.

Parameter	C	Q	F	F + Q
Absolute visceral fat (g)	7.07 ± 1.58	8.24 ± 2.07	6.66 ± 2.64	6.34 ± 2.77
Relative visceral fat (%)	2.09 ± 0.35	2.23 ± 0.43	2.22 ± 0.74	2.06 ± 0.59
Absolute epididymal fat (g)	2.15 ± 0.43	2.39 ± 0.35	1.69 ± 0.44	4.51 ± 8.03
Relative epididymal fat (%)	0.64 ± 0.09	0.65 ± 0.06	0.56 ± 0.14	1.37 ± 2.28

Data represented as mean ± standard deviation C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8.

3.4.2 Female rats

Table 3.6 shows the effects of orally administered quercetin on the adiposity of female Sprague Dawley rats fed a high fructose diet for eight weeks. No significant differences in the absolute and relative (to body mass) visceral fat mass were observed between treatment groups ($p > 0.05$).

Table 3.6: The effects of orally administered quercetin on the adiposity of female Sprague Dawley rats fed a high fructose diet for eight weeks.

Parameter	C	Q	F	F + Q
Absolute visceral fat (g)	8.39 ± 1.49	7.88 ± 1.43	8.26 ± 1.64	8.99 ± 2.75
Relative visceral fat (%)	3.63 ± 0.56	3.39 ±0.45	3.63 ± 0.57	3.88 ± 0.87

Data represented as mean ± standard deviation. C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8.

3.5 Effect of orally administered quercetin on serum insulin and adiponectin concentrations

3.5.1 Male rats

Table 3.7 shows the effects of orally administered quercetin on serum insulin and adiponectin concentrations of male Sprague Dawley rats fed a high fructose diet for eight weeks. No significant differences in serum insulin and adiponectin concentrations were observed between treatment groups ($p > 0.05$).

Table 3.7: The effects of orally administered quercetin on serum insulin and adiponectin concentrations of male Sprague Dawley rats fed a high fructose diet for eight weeks.

Parameter	C	Q	F	F + Q
Insulin (ng/mL)	1.25 ± 0.54	0.90 ± 0.74	0.94 ± 0.54	1.17 ± 0.45
Adiponectin (pg/mL)	64.01 ± 43.47	51.61 ± 43.21	57.72 ± 41.39	70.23 ± 32.54

Data represented as mean ± standard deviation. C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8

3.5.2 Female rats

Table 3.8 shows the effects of orally administered quercetin on serum insulin and adiponectin concentrations of female Sprague Dawley rats fed a high fructose diet for eight weeks. No significant differences in serum insulin and adiponectin concentrations were observed between treatment groups ($p > 0.05$).

Table 3.8: The effects of orally administered quercetin on serum insulin and adiponectin concentrations of female Sprague Dawley rats fed a high fructose diet for eight weeks.

Parameter	C	Q	F	F + Q
Insulin (ng/mL)	1.06 ± 0.52	1.13 ± 0.59	1.16 ± 0.66	0.80 ± 0.47
Adiponectin (pg/mL)	46.32 ± 34.02	63.31 ± 40.14	73.08 ± 47.16	74.25 ± 51.36

Data represented as mean ± standard deviation. C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8.

3.6 Effect of orally administered quercetin on serum biomarkers of renal and liver function

3.6.1 Male rats

Table 3.9 shows the effects of orally administered quercetin on serum biomarkers of renal (creatinine and BUN) and liver (ALT) function of male Sprague Dawley rats fed a high fructose diet for eight weeks. No significant differences in the serum creatinine and ALT concentrations were observed between treatment groups ($p > 0.05$). Rats receiving fructose ($p < 0.01$) and those receiving fructose + quercetin ($p < 0.05$) had significantly lower serum BUN concentrations compared to rats of the control group. Rats receiving fructose had significantly lower serum BUN concentrations than those receiving quercetin ($p < 0.05$). Rats in the fructose group and those in the fructose + quercetin group had a significantly lower BUN: Crea ratio than that of rats in the control group ($p < 0.05$).

Table 3.9: The effects of orally administered quercetin on serum biomarkers of renal and liver function of male Sprague Dawley rats fed a high fructose diet for eight weeks.

Parameter	C	Q	F	F + Q
Crea (mg/dL)	0.23 ± 0.05	0.27 ± 0.05	0.20 ± 0.07	0.20 ± 0.10
BUN (mg/dL)	21.13 ± 4.36^{a}	20.38 ± 3.38^{ac}	13.57 ± 5.44^{b}	15.13 ± 3.18^{bc}
BUN:Crea	91.43 ± 21.50^{a}	74.71 ± 11.77^{ab}	58.00 ± 22.49^{b}	49.00 ± 8.49^{b}
ALT (U/L)	104.30 ± 50.10	71.50 ± 22.03	82.38 ± 35.05	69.00 ± 13.49

Data represented as mean ± standard deviation. Values in a row with different superscripts are significantly different ($p<0.05$). C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8. Crea = Creatinine; BUN = Blood urea nitrogen; BUN: Crea = ratio of blood urea nitrogen to creatinine; ALT = Alanine aminotransferase.

3.6.2 Female rats

Table 3.10 shows the effects of orally administered quercetin on serum biomarkers of renal (creatinine and BUN) and liver (ALT) function of female Sprague Dawley rats fed a high fructose diet for eight weeks. No significant differences in the serum creatinine, ALT and BUN: Crea ratios were observed between treatment groups ($p > 0.05$). Rats receiving fructose + quercetin had significantly lower serum BUN concentrations than those receiving quercetin ($p < 0.01$). Rats receiving fructose ($p < 0.05$) and those receiving fructose + quercetin ($p < 0.01$) had significantly lower serum BUN concentrations compared to rats of the control group.

Table 3.10: The effects of orally administered quercetin serum biomarkers of renal and liver function of female Sprague Dawley rats fed a high fructose diet for eight weeks

Parameter	C	Q	F	F + Q
Crea (mg/dL)	0.26 ± 0.052	0.23 ± 0.08	0.26 ± 0.05	0.21 ± 0.07
BUN (mg/dL)	20.00 ± 3.21^{ac}	19.38 ± 3.85^{c}	15.50 ± 2.20^{bc}	13.50 ± 2.88^{b}
BUN:Crea	77.88 ± 14.54	81.17 ± 10.67	61.63 ± 15.67	67.86 ± 34.64
ALT (U/L)	51.63 ± 8.00	57.13 ± 19.79	64.25 ± 9.36	58.25 ± 24.36

Data represented as mean ± standard deviation. Values in a row with different superscripts are significantly different ($p < 0.05$). C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8. Crea = Creatinine; BUN = Blood urea nitrogen; BUN: Crea = ratio of blood urea nitrogen to creatinine; ALT = Alanine aminotransferase.

3.7 Effect of orally administered quercetin on liver mass

3.7.1 Male rats

Table 3.11 shows the effects of orally administered quercetin on the liver mass of male Sprague Dawley rats fed a high fructose diet for eight weeks. No significant differences in the absolute and relative (to body mass) liver mass were observed between treatment groups ($p > 0.05$)

Table 3.11: The effects of orally administered quercetin on the liver mass of male Sprague Dawley rats fed a high fructose diet for eight weeks.

Parameter	C	Q	F	F + Q
Absolute liver mass (g)	10.18 ± 0.91	10.71± 0.98	8.99 ± 1.12	9.13 ± 1.88
Relative liver mass (%)	3.03 ± 0.16	2.93 ± 0.14	3.00 ± 0.36	3.09 ± 0.18

Data represented as mean ± standard deviation. C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8.

3.7.2 Female rats

Table 3.12 shows the effects of orally administered quercetin on the liver mass of female Sprague Dawley rats fed a high fructose diet for eight weeks. No significant differences in the absolute and relative (to body mass) liver mass were observed between treatment groups ($p > 0.05$).

Table 3.12: The effects of orally administered quercetin on the liver mass of female Sprague Dawley rats fed a high fructose diet for eight weeks.

Parameter	C	Q	F	F + Q
Absolute liver mass (g)	7.15 ± 0.43	7.59 ± 1.18	7.61 ± 1.17	7.29 ± 0.71
Relative liver mass (%)	3.11 ± 0.29	3.28 ± 0.44	3.36 ± 0.44	3.20 ± 0.22

Data represented as mean ± standard deviation. C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8.

3.8 Effect of orally administered quercetin on liver steatosis

3.8.1 Male rats

3.8.1.1 Liver Steatosis scores in male rats

Figure 3.5 shows photographs of liver histological sections from representative rats from each dietary group. Subjectively, the steatosis observed in the groups receiving fructose alone and those receiving fructose + quercetin appeared more pronounced than that observed in the groups receiving quercetin alone and in the control group. Quantitatively, Table 3.13 shows the effects of orally administered quercetin on liver micro- and macro- steatosis scores of male Sprague Dawley rats fed a high fructose diet for eight weeks. Following objective assessment, no significant differences in the overall micro- and macro- steatosis scores were observed across all treatment groups ($p > 0.05$).

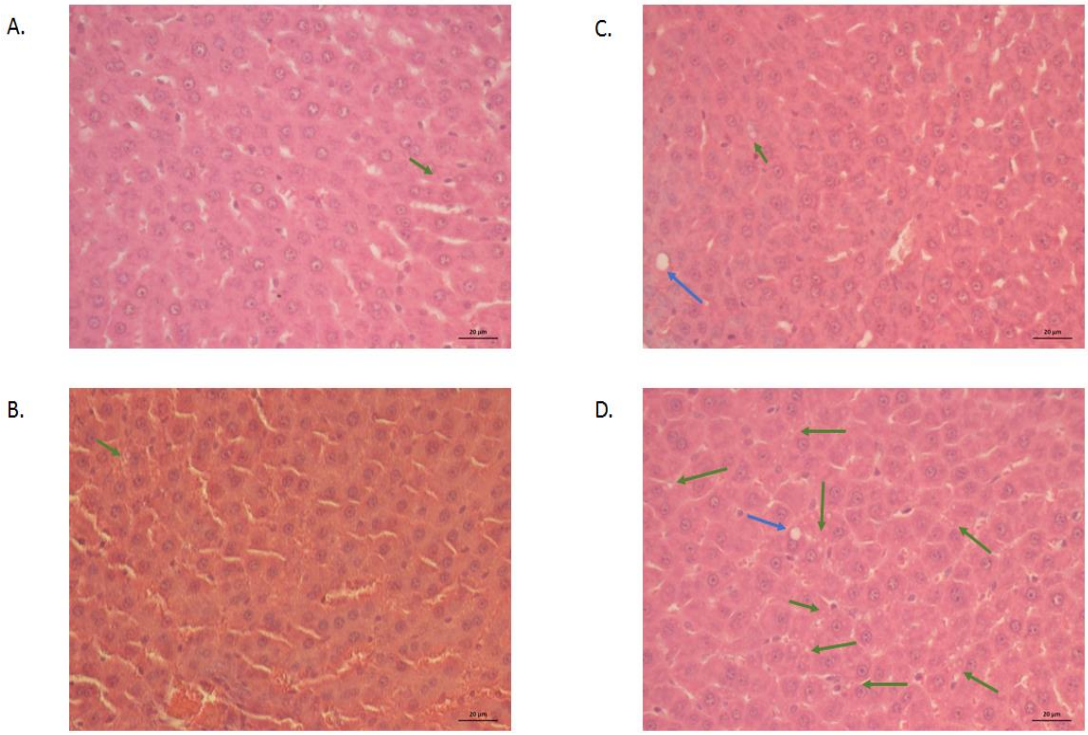

Figure 3.5: Liver histology sections (haematoxylin and eosin stain, 40 X magnification).
Images of the liver sections obtained from representative male Sprague Dawley rats after the eight week intervention period. The slides were stained with haematoxylin and eosin. (A) Control group (B) Quercetin group, (C) Fructose group, (D) Fructose + Quercetin group. Green arrows indicate micro-steatosis and blue arrows indicate macro-steatosis.

Table 3.13: The effects of orally administered quercetin on liver steatosis of male Sprague Dawley rats fed a high fructose diet for eight weeks.

Parameter	C	Q	F	F + Q
Micro-steatosis	0.0 (0.00 – 0.75)	0.50 (0.00 – 1.75)	1.00 (0.00 – 1.75)	2.00 (0.25 – 2.75)
Macro-steatosis	0.0 (0.00 – 0.00)	0.0 (0.00 – 0.00)	0.0 (0.00 – 0.00)	0.0 (0.00 – 0.00)

Data represented as median (interquartile range). C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8.

3.8.1.2 Percentage distribution of steatosis in male rats

To further explore the distribution of steatosis within and across the groups, the percentage distribution was determined. Table 3.14 shows the percentage of male Sprague Dawley rats with micro-steatosis after being fed a high fructose diet for eight weeks. A greater percentage of rats administered fructose with or without quercetin had micro-steatosis compared to the other groups.

Table 3.14: The effects of orally administered quercetin on the percentage distribution of micro-steatosis in male Sprague Dawley rats after being fed a high fructose diet for eight weeks.

	Micro-steatosis score			
Group	**0**	**1**	**2**	**3**
C	75	12.5	0	12.5
Q	50	37.5	12.5	0
F	37	37.5	25	0
F + Q	25	12.5	37.5	25

Data represented as percentage. C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8. Score 0 = < 5 % - minimal steatosis; Score 1 = 5 – 33 % - mild steatosis; Score 2 = 34 – 66 % - moderate steatosis; Score 3 = > 66 % - severe steatosis.

3.8.2 Female rats

3.8.2.1 Liver steatosis scores in female rats

Figure 3.6 shows photographs of histological sections from representative rats from each dietary group. Subjectively, steatosis appeared more pronounced in the groups receiving quercetin alone and fructose alone, compared to the control and fructose + quercetin groups.

Table 3.15 shows the effects of orally administered quercetin on liver micro- and macro- steatosis scores of female Sprague Dawley rats fed a high fructose diet for eight weeks. No significant differences in micro- and macro- steatosis scores were observed across all treatment groups ($p > 0.05$).

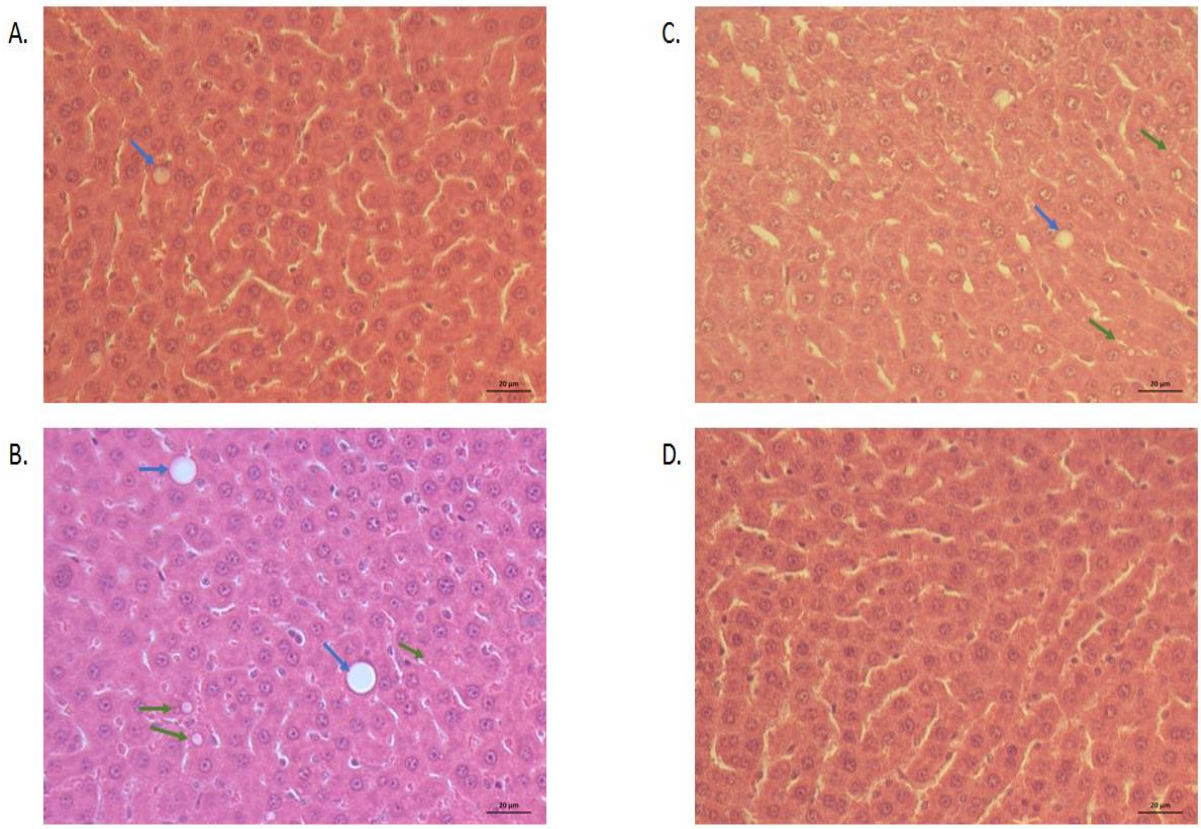

Figure 3.6: Liver histology sections (haematoxylin and eosin stain, 40 X magnification).
Images of the liver sections obtained from representative female Sprague Dawley rats after the eight week intervention period. The slides were stained with haematoxylin and eosin. (A) Control group. (B) Quercetin group. (C) Fructose group. (D) Fructose + Quercetin group. Green arrows indicate micro-steatosis and blue arrows indicate macro-steatosis.

Table 3.15: The effects of orally administered quercetin on liver steatosis of female Sprague Dawley rats fed a high fructose diet for eight weeks**.**

Parameter	C	Q	F	F + Q
Micro-steatosis	1.00 (0.25 – 2.75)	1.50 (1.00 – 3.00)	1.00 (0.25 – 2.75)	0.50 (0.00 – 2.00)
Macro-steatosis	0.0 (0.00 – 0.00)	0.0 (0.00 – 0.00)	0.0 (0.00 – 0.00)	0.0 (0.00 – 0.00)

Data represented as median (interquartile range). C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8.

3.8.2.2 Percentage distribution of steatosis in female rats

Table 3.16 shows the percentage of female Sprague Dawley rats with micro-steatosis after being fed a high fructose diet for eight weeks. Rats in the control group had varying degrees of steatosis. The quercetin only group had the greatest percentage of rats with severe steatosis (steatosis score 3).

Table 3.16: The effects of orally administered quercetin on the percentage distribution of micro-steatosis in female Sprague Dawley rats after being fed a high fructose diet for eight weeks.

	Micro-steatosis score			
Group	**0**	**1**	**2**	**3**
C	25	25	25	25
Q	12.5	37.5	12.5	37.5
F	25	37.5	12.5	25
F + Q	37.5	25	25	12.5

Data represented as percentage. C = Control group - tap water and plain gelatine cubes, n = 8; Q = Quercetin group - tap water and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8; F = Fructose group - 20 % fructose drinking solution and plain gelatine cubes, n = 8; F+Q = Fructose + Quercetin group - 20 % fructose drinking solution and gelatine cubes containing quercetin at 100 mg/kg body mass, n = 8. Score 0 = < 5 % - minimal steatosis; Score 1 = 5 – 33 % - mild steatosis; Score 2 = 34 – 66 % - moderate steatosis; Score 3 = > 66 % - severe steatosis.

CHAPTER 4: DISCUSSION

This study investigated the effects of dietary supplementation with quercetin on high fructose (20 % fructose drinking solution) induced metabolic derangements in growing male and female Sprague Dawley rat pups.

Fructose administration for eight weeks did not significantly affect, clinical biomarkers (fasting blood glucose, TGs, cholesterol), adiposity and serum insulin and adiponectin concentrations when compared to the negative control group. Fructose consumption did however compromise linear growth performance in both male and female rats. Although no significant differences in micro- and macro-steatosis scores were observed between treatment groups, fructose consumption seems to predispose male rats to the development of hepatic steatosis, since there were a greater percentage of male rats administered fructose with or without quercetin that had micro-steatosis compared to the other groups. Unlike in male rats, female rats in the fructose and fructose + quercetin group had a lower percentage of rats with severe steatosis. The quercetin only group in female rats had the greatest percentage of rats with severe steatosis. This could suggest that female rats treated with quercetin at an early age are more predisposed to the development of NAFLD later in life. Administration of quercetin alone did not affect clinical biomarkers or result in adverse health outcomes.

Body mass can be used as a measure for growth and is therefore often used as a surrogate measure for health in animals. Over or under nutrition can result in an increase or decrease in body mass gain. This is especially evident in rapid growth phases such as the early post-weaning phase (Johnson et al., 1973; Wainwright and Francey, 1987). In the current study it was found that there were no differences between treatment groups in body mass gain over the eight week study period which commenced from weaning to adolescence, phases which are characterised by rapid growth. This suggests that it was unlikely that the high fructose diet or treatment regimen provided had any negative effects on the health of the rats as measured by growth performance. However, body weight is acutely affected by various factors such as food intake and hydration status (Shirreffs, 2003) and as a result, long bone growth is often used as a more accurate measure for linear growth and growth performance than body mass (Ajibola, 2016; Bass et al., 2013; Felice et al., 2014).

During childhood and adolescence, bone formation exceeds bone resorption resulting in the acquisition of bone mass (Tjäderhane and Larmas, 1998; Tsanzi et al., 2008). With advancing age the reverse is applicable (Gómez-Cabello et al., 2012). Genetics, hormones, physical activity and diet are major determinants of peak bone mass (Levine, 2012; Yousef, 2015). Dietary approaches to avert bone loss have mainly focused on calcium consumption which is commonly acquired from the diet in animals and assists in calcium balance (Bass, et al., 2013; Tsanzi, Light, & Tou, 2008). Therefore compromised dietary intake of calcium from standard diet chow can result in reduced calcium intake. Meirelles et al., (2011) reported that animals fed a fructose rich diet tend to drink more fructose but consumed less rat fed. It is thus speculated that rats fed a fructose rich diet in the current study wherein feed intake was reduced could be responsible for the observed bone abnormalities. In this study it was observed that male rats receiving fructose with or without quercetin had lighter and shorter tibias and femora compared to control and quercetin only groups. Moreover, female rats receiving fructose + quercetin had shorter tibia and femora. It is also evident that early administration of quercetin in these rats did not protect against these fructose-induced adverse effects. This is contrary to a study by Oršolić et al. (2018) who reported that the antioxidant and estrogenic properties of quercetin administered at 100 mg/kg played an crucial role in the improvement of bone health by increasing bone weight index and bone mineral density in 60 day old 13cRA induced rat osteoporosis models. These differences may be due to the different experimental models used (osteoporosis previously vs high fructose diet in the current study). It can thus be concluded that although the high fructose diet had negative impacts on the bone indices, which may result in bone pathology later in life. Although, quercetin administration did not protect against the fructose induced adverse effects, administration of quercetin during the early developmental stages did not compromise the bones.

Although body mass can also be used as a measure of obesity, as previously mentioned body mass gain is influenced by other additional factors such as bone mass, fur/hair, and lean muscle. It is for that reason that visceral fat was measured to determine adiposity directly. Adiposity has traditionally been divided into two main components consisting of visceral fat which surrounds internal organs of the abdominal viscera and subcutaneous fat located under the skin (Yilmaz, 2012). Obesity is associated with an increased risk for the development of metabolic

derangements, the variations in fat distribution mediates the risk, with visceral fat more closely related to adverse risk profile than subcutaneous adipose tissue (Porter et al., 2009). Decreased glucose and insulin levels have been observed in humans, following the removal of visceral fat, whereas the removal of subcutaneous fat by liposuction did not improve glucose metabolism or lipid levels (Pontiroli et al., 2009; Porter et al., 2009). Increased depots of visceral fat are commonly associated with the development of metabolic derangements even when weight gain is similar amongst individuals (Gaggini et al., 2013; Maarman et al., 2016).

Fructose consumption has been reported to induce adverse health effects associated with visceral fat growth (O'Neill and O'Driscoll, 2015; Popkin and Hawkes, 2016) and thus increasing susceptibility to dyslipidaemia and its associated complications. Mamikutty et al*.,* (2014) reported consumption of 20 % fructose drinking solution in male Wistar rats over 8 weeks increased caloric intake and significantly increased visceral adiposity. However, in the current study we found that there was no significant difference in the visceral fat deposits between rats in the various treatment groups. Similarly, a study by Muhammad et al.,(2019) also found that there were no significant differences in the visceral fat deposits amongst female Sprague Dawley rats given 20 % fructose drinking solution and standard rat chow for 10-weeks after weaning at 21 days old. This may be due to the fact that rats were treated at a young age compared to previous studies, therefore it can be speculated that rats on a high caloric diet experienced an increase in metabolic rate which may be due to an increase in hypertrophy that results in increased heat production of brown adipose tissue. This in turn increased energy expenditure (Berry et al., 1985).

Despite the lack of change in visceral fat pad deposits that occurred in this study, other studies have reported that in pre-diabetics fructose can cause changes in the blood lipid profile (Schaefer et al., 2009). Prediabetes can also be characterized by alterations in glucose homeostasis, wherein the fasting glucose is elevated above normal ranges but it is not high enough to be considered as diabetes mellitus (Altemani et al., 2018). Alternatively prediabetes can be described by impaired glucose tolerance (Ferrannini et al., 2011; Tabák et al., 2012). In this study we found that there were no changes in fasting glucose concentrations amongst the treatment groups. However, normal levels of glucose can be a due to increased insulin concentrations as a result of insulin resistance (Altemani et al., 2018). No significant

differences in serum insulin concentrations as a result of the high fructose diet were observed in the current study. Therefore, it was unlikely that there was insulin resistance. Increased insulin levels are strongly associated with obesity (Elliott et al., 2002; Karpe et al., 2011; Porter et al., 2009) and the lack of observed visceral obesity may explain the findings.

Visceral obesity is associated with a decrease in circulating adiponectin concentrations (Falahi, et al., 2015; Lima et al., 2013). However, in this study it was found that adiponectin concentrations were normal. Adiponectin is an adipose derived protein whose secretion is decreased when there is an increase in visceral fat (López-Jaramillo et al., 2014; Ryo et al., 2004). It has been suggested that the activation of adiponectin receptors AdipoR1 and R2 result in increased lactate production in the liver and skeletal muscle, reduced hepatic gluconeogenesis, increased glucose uptake and inhibition of inflammation (Finelli and Tarantino, 2013). Hence, it is the suspected decrease in hepatic glucose output that contributes to the regulation of whole body glucose homeostasis by increasing muscle uptake of glucose and reducing hepatic gluconeogenesis (Robinson et al., 2011). Thus adiponectin can promote insulin action and ameliorate insulin resistance thus making adiponectin an insulin sensitizer (Kadowaki and Yamauchi, 2005; Trujillo and Scherer, 2005). Therefore, the lack of differences in adiponectin concentrations could be related to the lack of differences in the visceral fat deposits and fasting glucose concentrations. Increased visceral obesity and metabolic syndrome were previously thought to result in the development of NAFLD. However, it has been shown that NAFLD can occur without the development of obesity or changes in lipid profiles (Abdelmalek et al., 2010). Indeed, in the current study, the interventions did not result in differences in the TGs, LDL and HDL beyond normal ranges reported for rats.

Upon assessing the liver histology, it was found that although there was no significant difference in the overall hepatic micro- or macro- steatosis scores amongst treatment groups, fructose consumption was associated with an increased percentage of male rats with micro-steatosis. Of potential concern was the observation that quercetin administration in female rats alone during the early post weaning period increased the percentage of rats presenting with micro-steatosis. It can therefore be speculated that the administration of quercetin for two weeks, during the early post-weaning period predisposes female rats to the development of micro-

steatosis. These findings were different from those observed by Kim et al., (2015) where six-week old male C57BL/6 mice fed a high fat diet (lard and soybean oil 60 %; carbohydrate 20 %; protein 20 %) and treated with quercetin (100 mg/kg) for nine-weeks had reduced obesity induced hepatic lipid accumulation. The authors attributed the reduced hepatic lipid accumulation to quercetin inducing HO-1 via the Nrf-2 pathway, thus enhancing mitochondrial oxidative capacity. Unfortunately these pathways were not explored in the current study, due to technical limitations.

Although the potential health benefits of administering phytochemicals such as quercetin have previously been reported, potential harmful effects of these ethnomedicines also need to be determined if they are to be considered as alternative interventions in the management of diseases. There is limited research on the safety and efficiency of most plant derived medicines in the treatment and/or prevention of diseases (Bode and Dong, 2015; Brima, 2017; Namjoo et al., 2013). The WHO estimates that 80 % of populations in Africa and Asia use medicinal plants (Mahapatra et al., 2019). Despite the perceived benefits of medicinal plants some of them have unpleasant effects that may lead to acute toxicity and death in patients and although many ethnomedicines have been shown not to be toxic, dose and duration of treatment need to be considered to prevent potential harm (Tamokou and Kuete, 2014). Therefore, the assessment of surrogate biochemical markers of health was investigated to determine if administration of quercetin had any toxic health effects on the renal and hepatic systems. The liver and kidneys contain enzymes responsible for drug metabolism and the biotransformation of chemicals (Dixon et al., 2014; Zgheib and Branch, 2017). Therefore the administration of toxic substances could result in liver and kidney damage (Anders, 1980; Remmer, 1970; Tarloff et al., 1987). In this study we found that no significant differences were observed in the biochemical surrogate markers of liver and kidney function measured between treatment groups.

The consumption of a fructose rich diet has been associated with elevated activity of alanine transaminase (ALT), a liver enzyme that serves as a marker for liver damage therefore its serum concentration is used to assess hepatotoxicity (Aulbach and Amuzie, 2017; Liu et al., 2014). Some studies have found that serum concentration of ALT, although suggestive of liver disease, does not always indicate the absence of significant liver disease or other components of MetS in rodent models and human

epidemiologic studies (Kang et al., 2011; Perés Wingeyer et al., 2008). In the current study serum ALT levels remained normal, this may be due to the lack of severe liver damage as noted by the similar hepatic steatosis scores observed. The liver has a large residual functional capacity and as a result there needs to be more than 50 % damage before liver function is compromised (Tucker and Heaton, 2005). ALT is located in the cytoplasm of hepatocytes, where fat deposition usually occurs. The lack of increase in serum ALT levels may be due to the fact that the fat deposition was not extensive enough to alter hepatocyte cell membrane integrity which would result in cytoplasmic leakage of ALT into the blood stream. Therefore, it can be assumed that although the liver has incurred some damage, it was not substantial enough to induce an elevation in serum ALT levels.

Renal function markers creatinine and BUN were also assessed to determine renal function. Previous studies have stated that creatinine is a more specific indicator of renal glomerular filtration rate than BUN that is affected by non-renal factors such as hydration status (McMenemey, 1948; Zachariah et al., 2019). Determination of the BUN to serum creatinine ratio is a useful computation in diagnosing suspected renal disorders (Thomas et al., 2017; Xue et al., 2014). In this study we found that there was no significant difference in the serum creatinine levels of both male and female rats. However, although it was observed that serum BUN levels did not exceed normal ranges for rats, it was observed that both male and female rats fed a fructose rich diet had significantly lower levels of BUN than rats in the control group. This may be because it has been observed that rats on a high fructose diet will consume more fructose and decrease their food intake (Tsanzi, et al., 2008). As a result, less protein is consumed therefore rats have lower protein turnover, thus resulting in low serum concentrations of BUN.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Diseases of lifestyle related to obesity and metabolic dysfunction often require lifelong therapeutic management which is costly and places a heavy burden on health care delivery systems. Targeted strategic interventions which could provide long term prophylactic potential are advocated for global health improvement. There are several periods of developmental plasticity in which interventions can have long lasting health outcomes. Previous studies have focused on the peri-conceptual, foetal, and early phases of life as targets of intervention. This study investigated whether quercetin administration in the early post-weaning period would prevent the development of negative health outcomes associated with high dietary fructose in male and female Sprague Dawley rats, later in life. The novelty of this study is that it has focused on the early post-weaning period as a strategic target for intervention for long lasting positive health outcomes.

High dietary fructose feeding is a recognised and established model for metabolic dysfunction. However, in the current study administration of 20 % fructose ad libitum for eight weeks from weaning did not cause the overt development of aspects of metabolic syndrome such as altered growth performance, increased adiposity and deleterious changes in surrogate biomarkers of health.

The administration of quercetin during the early post weaning period did not impact linear growth, body mass or markers of general health. However, the administration of quercetin alone seems to have predisposed female rats to the development of hepatic steatosis. These observations suggest that quercetin when administered during the early post-weaning period could result in NAFLD in adulthood. Of note in this study is the sexually dimorphic response of the rats to fructose administration and their susceptibility to hepatic steatosis development.

Thus, in conclusion this study has highlighted the importance of considering both sexes and highlighted the fact that liver steatosis can develop independently of other generalised metabolic disorders.

5.2 Limitations and recommendations

One of the key elements of this study's hypothesis was premised on the previously reported change in digestive and metabolic enzymatic activity associated with weaning, therefore for future studies I recommend assessment of the gene expression of lipogenic enzymes which are expressed for the first time in the adipose tissue and liver during the post-weaning period and an assessment of pancreatic enzyme expression that occurs during weaning. Investigating the expression of these enzymes could help in further understanding the effect of quercetin on adiposity and NAFLD at a molecular level.

Although the study intervention period was based on previous studies (8 weeks) which used older rats, future studies with young rats could be extended for longer periods of time, since previous studies have shown increased incidence of metabolic derangements following fructose feeding periods longer than eight weeks. It would be insightful in future studies to use different quercetin doses in order to determine a dose dependent response (if any).

CHAPTER 6: REFERENCES

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APPENDICES

APPENDIX A: Ethical Clearance Certificates

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2017/02/07/B

APPLICANT: Dr J Donaldson

SCHOOL: School of Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Quercetin exposure pre- and post-weaning in the prevention and treatment of diet-induced metabolic syndrome in growing Sprague Dawley rat pups

Number and Species

192 each male and female postnatal day 7, 36 female postnatal day 21 and 40 female nursing adult Sprague Dawley rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 2017/02/28. This approval remains valid until 2019/03/15.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None

Signed: _____ (Chairperson, AESC) Date: 22/03/2017

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 19/03/17

cc: Supervisor: N/A
Director: CAS

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AESC 2018

Please note that only typewritten applications will be accepted. Should additional space be required for section "I" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Dr Janine Donaldson

b. Department: School of Physiology

c. Experiment to be modified / extended **AESC NO: 2017/02/07/B**

Other M&E's : 2

d. Project Title: Quercetin exposure pre- and post-weaning in the prevention and treatment of diet-induced metabolic syndrome in growing Sprague Dawley rat pups.

e.	Number and species of animals **originally approved**:		192 male and 192 female Sprague Dawley rat pups-postnatal day 7; 36 female Sprague Dawley rat pups-postnatal day 21; 40 female nursing adult Sprague Dawley rats
f.	Number of additional animals **previously allocated on M&Es**:		N/A
g.	Total number of animals **allocated** to the experiment to date:		36 female Sprague Dawley rat pups-postnatal day 21 and 38 male Sprague Daley rat pups-postnatal day 21
h.	Number of animals **used** to date:		36 female Sprague Dawley rat pups-postnatal day 21 and 38 male Sprague Daley rat pups-postnatal day 21

i. Specific modification / extension requested:

- We would like to modify the study design (outlined as Experiment 1 in the originally approved ethics protocol) as follows:
  - We want to **begin the interventions/treatments during the early postweaning phase instead of during the neonatal phase** (days 7-21) and continue the treatments for two weeks following weaning (days 21-35). Thus there will be no gavaging of the rat pups prior to weaning and the study will commence on postnatal day 21.
  - We would also like to **extend the fructose feeding phase** such that the groups receiving fructose will undergo 12 weeks in total of fructose feeding (two weeks during the treatment phase, days 21-35 and then 10 weeks following the treatment phase, days 35-105).
  - We would also like to **remove the fenofibrate-treatment group and reduce the overall number of rats** required. Instead of making use of 96 male and 96 female, 7-day old Sprague Dawley rat pups, we will only make use of **40 male and 40 female Sprague Dawley rat pups – postnatal day 21** (after weaning).

- We will also **not be required to split the groups into two following the treatment period and in doing so we will also be reducing the overall number of rats required.**

- We would like to include the following people as co-workers on the project:
  - Ramatsobane Rozette Tladi (707338) (Miss Ramatsobane Rozette Tladi is an MSc student and will use the data for her master's project)
  - Nasiru Muhammad (co-worker)
  - Kgomotso Motshoane (co-worker)
  - Lihle Goba (co-worker)
  - Thobekile Dladla (co-worker)

j. Motivation for modification / extension:

- The overall aim of my study is to investigate the effect of interventions (i.e. quercetin treatment) during critical phases of development on metabolic health outcomes. The periconceptual, gestational, neonatal and early postweaning phases are recognized as windows of developmental plasticity, during which exposure to stress, altered nutrition, chemicals, drugs or infection, could lead to functional changes which predispose the individual to development of disease later in life (Heindel and Vandenberg, 2015). These periods of plasticity can also serve as times where improved health in later life can be promoted, reducing the individual's susceptibility to disease (Heindel et al., 2016). Thus the proposed study design aims to investigate the early postweaning phase as a target of interest instead of the neonatal phase. This modification will make the administration of quercetin to the rats a lot less challenging for my MSc student and at the same time since we will no longer be gavaging the rat pups (prior to weaning) they will not be unnecessarily stressed.

- Following preliminary results from our lab, where significant fructose-induced metabolic dysfunction has only been observed following at least 8 weeks of fructose diet feeding, we would like to extend the feeding period to a total of 12 weeks. The first two weeks (phase 1) will serve as an intensive treatment period, where groups will receive gelatine cubes (either plain or containing 100 mg/kg quercetin) twice daily. Thereafter, phase 2 will commence and continue for 10 weeks, during which the groups will continue receiving normal rat chow and fructose solution/tap water (depending on the group), as previously approved but for a longer duration in the modified study.

- The focus of the study is on quercetin. Thus in modifying the study, the fenofibrate (positive control) group will be removed. We will make use of **4 experimental groups with 20 rats in each group (10 males and 10 females) which will be fed their respective diets for a period of 12 weeks in total (2-week treatment period and 10 weeks continuation of normal rat chow and fructose solution, but no treatment)** (please see Figure 1 and Figure 2 below, detailing the newly proposed groups and experimental timeline (Fig 1) versus the original groups and experimental timeline (Fig 2)).

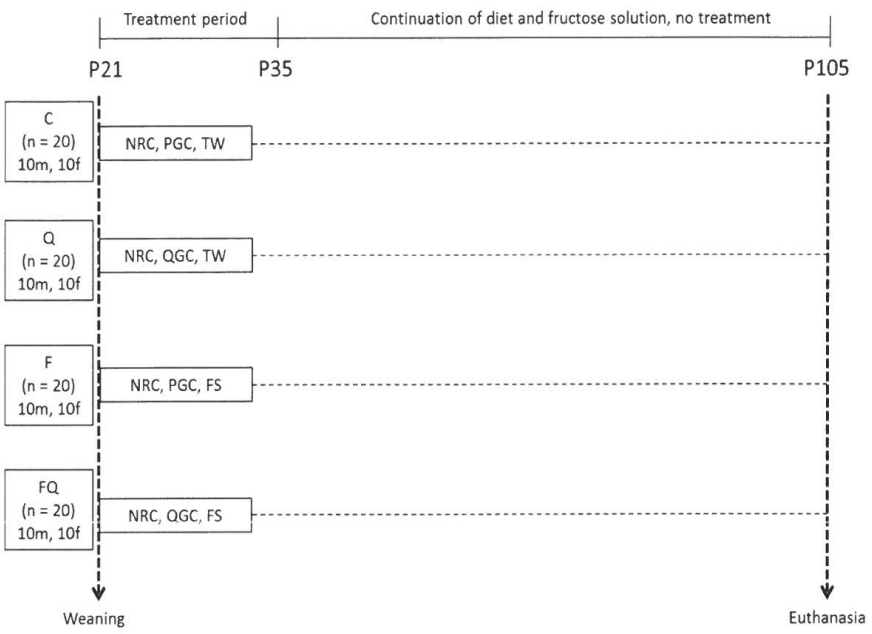

Figure 1.

- C (Control) - receive normal rat chow, plain gelatine cubes and tap water to drink
- Q (quercetin) - receive normal rat chow, gelatine cubes containing quercetin and tap water to drink
- F (high fructose) - receive normal rat chow, plain gelatine cubes and fructose in drinking water
- FQ (high fructose – quercetin) - receive normal rat chow, gelatine cubes containing quercetin and fructose in drinking water

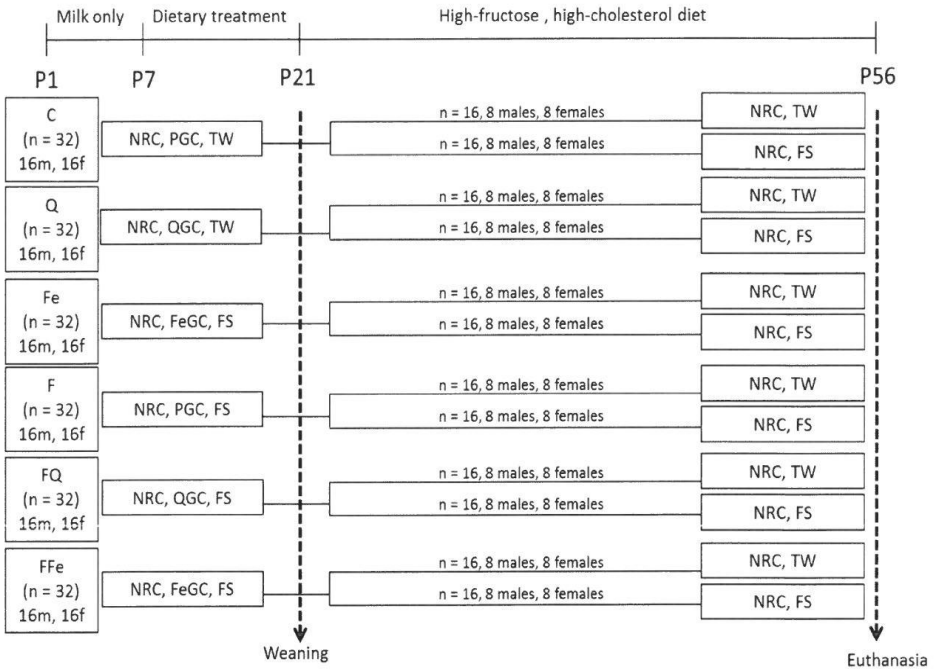

Figure 2.

- C (Control) - receive normal rat chow, plain gelatine cubes and tap water to drink
- Q (quercetin) - receive normal rat chow, gelatine cubes containing quercetin and tap water to drink
- Fe (fenofibrate) - receive normal rat chow, gelatine cubes containing fenofibrate and tap water to drink
- F (high fructose) - receive normal rat chow, plain gelatine cubes and fructose in drinking water
- FQ (high fructose – quercetin) - receive normal rat chow, gelatine cubes containing quercetin and fructose in drinking water
- FFe (high fructose – fenofibrate) - receive normal rat chow, gelatine cubes containing fenofibrate and fructose in drinking water

References:

Heindel, JJ; Balbus, J; Birnbaum, L; Brune-Drisse, MN; Grandjean, P; Gray, K; Landrigan, PJ; Sly, PD; Suk, W; Slechta, DC; Thompson, C and Hanson, M (2016). Developmental origins of health and disease: Integrating environmental influences. Endocrinology **2016**: 17-22.

Heindel, JJ and Vandenberg, LN (2015). Developmental origins of health and disease: A paradigm for understanding disease etiology and prevention. *Current Opinion in Pediatrics* **27**: 248-253.

Signature: Date: 09/05/2018

Dr Janine Donaldson

RECOMMENDATIONS:

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Signature: ..... Date: MAY 15 2018

Chairman, AESC