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THE PROPHYLACTIC EFFECT OF 4-HYDROXYISOLEUCINE DERIVED FROM FENUGREEK SEEDS AGAINST HIGH FRUCTOSE DIET INDUCED METABOLIC DYSFUNCTION IN GROWING RATS

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, School of Physiology, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 2019

DECLARATION

I, Kgomotso Cathrine Motshoane hereby declare that this dissertation is my own work, unaided work. It is being submitted for the degree of Master of Science in Medicine in the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……

Kgomotso Cathrine Motshoane

Signed on the……..day of…….20…….at…….

DEDICATION

To the everlasting memory of my mother and father

- **Late Leah Mokete Motshoane**

(1965-2008)

- **Late Joel Kwena Motshoane**

(1961-1999)

You have taught me the value of education and I've come this far because of your unforgotten wishes for my success. Continue to rest in harmony.

PRESENTATIONS ARISING FROM THIS RESEARCH PROJECT

Poster presentations

1. **Motshoane, K.C., Olateju, O.I., Nkomozepi, P., Erlwanger, K.H.** The prophylactic effect of 4-hydroxyisoleucine against high-fructose diet-induced Non-alcoholic fatty liver disease in growing rats (P2). 47th Congress of the Physiology Society of Southern Africa hosted by the Walter Sisulu University at the East London International Conventional Centre from the 18th-21st August 2019.

2. **Motshoane, K.C., Olateju, O.I., Nkomozepi, P., Erlwanger, K.H.** 4-Hydroxyisoleucine as prophylaxis against high-fructose diet-induced Non-alcoholic fatty liver disease in growing rats (HS30). 10th Cross Faculty Postgraduate Symposium hosted by the University of the Witwatersrand at Johannesburg from the 3rd-4th September 2019.

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ABSTRACT

Increased fructose consumption is associated with the development of obesity, dyslipidaemia, diabetic nephropathy and non-alcoholic fatty liver disease (NAFLD). These conditions place a heavy burden on healthcare delivery systems. Phytochemicals are being investigated as alternative management interventions for metabolic disorders. To investigate whether orally administered 4-Hydroxyisoleucine (4-OH-Ile) (derived from fenugreek seeds) could prevent the development of high-fructose-induced metabolic dysfunction in growing rats, eighty weaned rats were grouped (8 males and 8 females per group). In addition to standard rat chow, group I had plain drinking water (PDW); II had 20% fructose solution (20%FS); III had 20%FS+4-OH-Ile (500 mg/kg); IV had PDW+4-OH-Ile; V received 20%FS+Fenofibrate (100 mg/kg) for 10 weeks. In both sexes, there were no significant differences in visceral fat mass and plasma glucose, adiponectin and long bone growth. Diabetic nephropathy histologic assessment showed that females administered 20%FS alone or with Fenofibrate had smaller renal corpuscle areas ($p=0.01$) compared to controls. All males had smaller renal corpuscles ($p=0.0001$) compared to those on the normal diet. NAFLD assessment showed that females on the high fructose diet had greater macrovesicular steatosis compared to all other females and greater microvesicular steatosis scores ($p=0.01$) compared to females administered PDW+4-OH-Ile and 20%FS+Fenofibrate. There were no significant differences in the NAFLD scores of males ($p>0.5$). There were notable sexually dimorphic responses to the high fructose diet. The 4-OH-Ile was able to prevent the development of diet induced NAFLD in males however 4-OH-Ile was unable to prevent the development of diabetic nephropathy in both sexes.

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ABBREVIATIONS

α: Alpha

β: Beta

ɤ: Gamma

∑p: Sum of points

%: Percentage

20%FS: 20% Fructose solution

4HGC: 500 mg/kg body weight of 4-hydroxyisoleucine (4-OH-Ile) in a gelatine cube

4-OH-Ile: 4-Hydroxyisoleucine

AESC: Animal Ethics Screening Committee

AMPK: Adenosine monophosphate-activated protein kinase

ANOVA: Analysis of variance

AREC: Animal Research Ethics Committee

ATP: adenosine triphosphate

A: Area

A_R: Area of renal corpuscle

A_B: Area of Bowman's capsule space

A_G: Area of glomerulus

A_P: Area per point

$A_{fraction}$: Area fraction

BW: Body weight

CAS: Central animal services

DN: Diabetic nephropathy

ELISA: Enzyme linked immunosorbent assay

FENGC: 100 mg/kg body weight of Fenofibrate in a gelatine cube

FF: Fenofibrate

GH: Growth hormone

GIT: Gastrointestinal

GLUT: Glucose transporter

GMO: Genetically modified organism

HDL: High-density lipoprotein

H&E: Haematoxylin and Eosin

HOMA-IR: Homeostatic model of insulin resistance

IL: Interleukin

LDL: Low density lipoprotein

MCH: Mean cell haemoglobin

MT: Masson's Trichrome

NAS: Non-alcoholic fatty liver disease activity score

NASH: Non-alcoholic steatohepatitis

NAFLD: Non-alcoholic fatty liver disease

OD: Optical density

PDW: Plain drinking water

PGC: Plain gelatine cube

PI-3-K: Phosphoinositide 3-kinases

PPAR: Peroxisome proliferator activated receptor SD: Standard deviation

ROS: Reactive oxygen species

RPM: Revolutions per minute

SRC: Standard rat chow

SREBP: Sterol regulatory element binding protein

STZ: Streptozocin

SD: Standard deviation

STD: Standard

TG: Triglycerides

TZD: Thiazolidinediones

Tcholesterol: Total cholesterol

Ttriglyceride: Total triglyceride

VLDL: Very low density lipoprotein

CHAPTER 1: LITERATURE REVIEW AND RATIONALE

1. Introduction /Literature review

1.1 Obesity

Obesity, which is excess accumulation of fat in the body, has become a global health problem due to its contribution to increased morbidity and mortality rates worldwide (Bekar et al., 2014; Uzogara, 2017). Between 1980 and 2015, the prevalence of obesity has doubled globally (Figure 1.1) such that about a third of the global population is now considered overweight or obese (Chooi et al., 2019). Reports have also shown that about 609 million adults >20 years old were estimated to be obese in 2015 (Chooi et al., 2019). Furthermore, epidemiological data anticipates that approximately 21% of women and 18% of men worldwide will be obese by the year 2025 (Pereira et al., 2017).

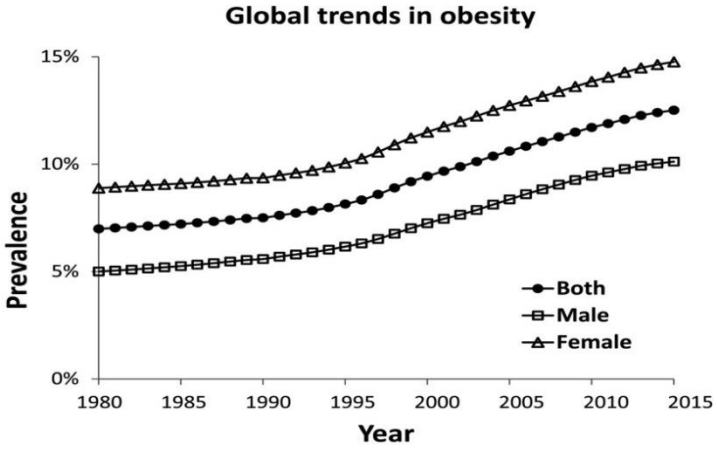


Figure 1. 1: Age-standardised global prevalence of obesity in men and women ≥20 years old by year (ca. 1980–2015). Source: (Chooi et al., 2019)

1.1.1 Childhood obesity

Childhood obesity has also increased significantly worldwide and is now considered a greater global health problem than adulthood obesity (Cho et al., 2015). Due to this, childhood obesity has become a focus of attention as it tends to persist into adulthood resulting in increased mortality and morbidity later in life (MacPherson et al., 2016). The prevalence of childhood obesity has also doubled between 1980 and 2015 (Weihrauch-Blüher et al., 2019). Approximately 107.7 million children were obese worldwide in 2015 which on average is 23% of the global population (Weihrauch-Blüher et al., 2019). Furthermore, the increase in childhood obesity has also caused an increase in the prevalence of metabolic syndrome in

young children suggesting a link between childhood obesity and metabolic syndrome (Friend et al., 2013).

1.2 Metabolic syndrome

Metabolic syndrome is defined as a cluster of disorders such as visceral fat accumulation, type 2 diabetes, insulin resistance, non-alcoholic fatty liver disease (NAFLD) and dyslipidaemia (Srikanthan et al., 2016; Ter Horst and Serlie, 2017). These metabolic diseases increase the risk of developing other diseases such as cardiovascular diseases and diabetic nephropathy (Tappy and Le, 2010). Historically, metabolic syndrome was considered an adult problem however recent studies have shown that young children have been identified with metabolic syndrome (Friend et al., 2013). As research has shown that childhood obesity persists into adulthood, there is a need to identify the causes of obesity and metabolic syndrome in young children (Taha Doris et al., 2008).

1.3 Causes of obesity and its associated metabolic disorders

Several factors contribute to the problem of obesity and its associated metabolic disorders (Zimmet et al., 2007). Among the multiplicity of factors, poor dietary patterns have been reported as the major cause of obesity especially in urban settlements (Zimmet et al., 2007). Research shows that a majority of the South African black population is exposed to a market economy which has caused the shift from traditional foods (which are low in fats and processed sugars) to foods which are high in fats such as fast foods (Kruger et al., 2005). In addition, sociocultural influence can drive a desire for body fat. For example, in some African cultures, a large body size in women and children is indicative of healthy living and richness that these women are proud to portray to the community and which also symbolizes the economic status of their husband (Walker et al., 2001; Eam, 2011). This cultural perception is a major challenge in managing obesity in these communities (Walker et al., 2001; Eam, 2011). Diets such as the increased consumption of fructose have also been shown to increase accumulation of body fat which directly contributes to increased prevalence of obesity and its associated metabolic disorders (Srikanthan et al., 2016).

1.3.1 Fructose

Fructose is a monosaccharide that is highly lipogenic (Vos and Lavine, 2013; Basaranoglu et al., 2015). It is sweeter than glucose or sucrose and is frequently added to processed beverages and foods in high quantities throughout the world (Vos and Lavine, 2013; Basaranoglu et al., 2015). The consumption of fructose has increased worldwide by approximately 30% over the past 40 years (Pereira et al., 2017). The consumption of fructose in countries like the USA has increased by approximately 1000% between the years 1970 and 1990 and it continues to increase exponentially (Carvalho et al., 2018; Tappy and Lê, 2010).

The consumption of fructose has several negative effects. For example, fructose has a low impact on satiety which results in increased consumption of food (Pereira et al., 2017). While glucose triggers the release of insulin by pancreatic beta cells, fructose is not absorbed into the pancreas due to the lack of glucose transporter 2 (GLUT2) and glucose transporter 5 (GLUT5) and thus cannot stimulate the release of insulin (Pereira et al., 2017). The lack of insulin stimulation by fructose results in poor glycaemic control which could lead to development of diseases e.g. diabetes mellitus. Fructose also does not stimulate the release of gastric inhibitory peptide which is known to induce secretion of insulin (Tran et al., 2009). Fructose also inhibits hepatic lipid oxidation thus enhances the synthesis of very-low-density lipoproteins (VLDL)-triglycerides and fatty acid re-esterification (Rizkalla, 2010). Phosphofructokinase, a rate limiting enzyme, promotes glycogenesis, insulin secretion and inhibits the formation of triglycerides from glucose (Woo et al., 2010). On the contrary, fructose does not stimulate the activity of phosphofructokinase (Woo et al., 2010).

1.3.1.1 Metabolism of fructose

Fructose is absorbed across the epithelial barrier into the bloodstream and cells by fructose specific GLUT5 (Yilmaz, 2012). The absorbed fructose is then transported to the liver via the hepatic portal vein (Yilmaz, 2012). Unlike glucose, fructose absorption from the intestines is independent of sodium absorption and ATP hydrolysis thus resulting in an excess uptake of fructose by the liver (Rizkalla, 2010). Due to absence of GLUT 5 transporters in the pancreas and other organs, fructose is exclusively metabolised in the liver by fructokinase (Tran et al., 2009; Rizkalla, 2010). In the liver fructose is phosphorylated into fructose-1-phosphate by ketohexokinase (Ter Horst and Serlie, 2017). The fructose-1-phosphate is then split into two triose phosphate intermediaries namely glyceraldehyde-3-phosphate and dihydroxyacetone

phosphate by activities of aldolase B and triokinase (Angelopoulos et al., 2009; Ter Horst and Serlie, 2017; Yilmaz, 2012). The dihydroxyacetone phosphate ultimately forms the backbone of triglycerides while glyceraldehyde-3-phosphate produces the acetyl-CoA units needed for *de novo* lipogenesis and synthesis of fatty acids (Angelopoulos et al., 2009). The breakdown of fructose is much faster than that of glucose because it bypasses the rate-limiting step of glycolysis (Rizkalla, 2010; Ter Horst and Serlie, 2017). Given that fructose is a major cause of metabolic syndrome, there is a need to further analyse the different components of the disease (metabolic syndrome).

1.4 Components of metabolic syndrome

1.4.1 Adiposity

Body fat depots are classified into two categories namely subcutaneous fat which is located under the skin and visceral fat which surrounds abdominal viscera (Yilmaz, 2012). Visceral fat is more metabolically harmful than subcutaneous fat thus the presence of excess visceral fat is of concern (Yilmaz, 2012). The excessive consumption of fructose causes hypertrophy of white adipose tissue which triggers an increase in secretion of inflammatory cytokines adipokines (Pereira et al., 2017b). Inflammatory cytokines such as interleukin 6 (IL-6) have negative effects because they bind to cellular receptors and activate metabolic cascades that perpetuate insulin resistance (Pereira et al., 2017b). Some adipokines such as adiponectin, exclusively secreted by the adipocytes and derived from white adipose tissue, have properties of health benefit e.g. anti-atherosclerotic and anti-inflammatory properties (Lara-Castro et al., 2007; Tran et al., 2009). Adiponectin activates peroxisome-proliferator activating receptors gamma (PPARγ) which are involved in the metabolism of lipids and glucose (Lara-Castro et al., 2007; Oidor-Chan et al., 2016). In addition, it causes an increase in Adenosine monophosphate-activated protein kinase (AMPK) activity in muscle and liver which helps to improve glucose tolerance (Lara-Castro et al., 2007; Oidor-Chan et al., 2016). It is also responsible for insulin sensitization that helps in protecting against development of type 2 diabetes (Ryo et al., 2010; Tran et al., 2009). Despite the health benefits of adiponectin, the increased accumulation of white adipose tissue has been shown to be associated with the development of other diseases such as diabetes mellitus (Metwally et al., 2017; Yilmaz, 2012).

1.4.2 Diabetes mellitus

Hyperglycaemia or increased fasting blood glucose levels are indicative of diabetes mellitus (Barros et al., 2007). There are two types of diabetes mellitus, namely type 1 and type 2. The latter is classified as a metabolic disorder characterized by either a defect in insulin action or a defect in insulin secretion, or occurrence of both (Barros et al., 2007). The prevalence of type 2 diabetes is increasing in young children worldwide (Simsek et al., 2010). Type 2 diabetes accounts for about 90% of the diabetes population (Barros et al., 2007; Son et al., 2015; Zafar and Gao, 2016). Insulin resistance is present in approximately 90% of patients with type 2 diabetes, indicating that insulin resistance is the main cause of type 2 diabetes (Barros et al., 2007; Son et al., 2015; Zafar and Gao, 2016). In the long term, diabetes mellitus causes dysfunction, failure and damage of vital organs such as kidneys resulting in diabetic nephropathy (Barros et al., 2007).

1.4.2.1 Diabetic nephropathy

Diabetic nephropathy (DN) is the loss of kidney functions as a result of loss of the glomerular filtration capabilities (Muhammad and Nazar, 2014). DN can affect renal nephron microvasculature. Metabolic syndrome, particularly associated type 2 diabetes, has been shown to contribute to development of DN (Abdel-Kawi et al., 2016). Although the major risk factors for development of DN include poor glycaemic control and dyslipidaemia, which are prominent in diabetes, DN pathology is not exclusive to diabetic patients (Lim, 2014). Hypertension, smoking, obesity and genetics are also risk factors for development of complications similar to DN. Some of the morphological lesions characterising DN are observed in renal glomeruli and include thickening of the glomerular membrane and diffuse mesangial expansion which is also known as glomerulosclerosis (Rockwell, 1994). Mesangial expansion and glomerular membrane thickening are a result of extracellular matrix accumulation with types IV and VI collagen (Kim et al., 1991). These complications develop in approximately 40% of type 2 diabetes patients (Alicic et al., 2017). It has been reported that 50 out of 82 type 2 diabetic patients develop nephropathy characterized by mesangial expansion (Brito et al., 1998). Nonetheless, renal corpuscle size may be affected by high-caloric diets e.g. high-fructose diets which cause kidney swelling resulting in increased renal corpuscle size (Kretowicz et al., 2011). Nephrotoxicity on the other hand can cause a decrease in renal corpuscle size (Kizhner and Werman, 2002; Saleh et al., 2017). As yet, there is no

treatment or intervention for diabetic nephropathy. However, intensive glycaemic control or management reduces the risk of progression to diabetic nephropathy by ensuring that there is adequate insulin secretion or there is no insulin resistance (Alicic et al., 2017).

1.4.3 Insulin resistance

Two of the most common functions of insulin include maintaining sufficient energy stores and regulating plasma glucose concentrations (Porte, 2006). When cells fail to respond to the stimulatory effects of insulin then it is regarded as insulin resistance (Basaranoglu et al., 2015). Adipokines secreted from adipose tissue are reported to be linked to the development of insulin resistance as they act as paracrine, autocrine or endocrine agents and induce hepatic insulin resistance by interfering with insulin pathways (Li et al., 2013). The main site of insulin action is the liver which is the reason why hepatic insulin resistance is important and has an effect on the whole body (Li et al., 2013). Although hepatic insulin resistance results in increased hepatic glucose production and decreased glycogen synthesis thus causing hyperglycaemia but insulin resistance may be present in the absence of hyperglycaemia (Leclercq et al., 2007; Simsek et al., 2010). It has been shown that with insulin resistance, there is a shift in lipid metabolism which is characterised by increased triglyceride synthesis, peripheral lipolysis and the uptake of free fatty acids by the liver (Armiliato, 2015). Excessive uptake of triglycerides by the liver induces free fatty acid β-oxidation in the mitochondrial and promotes lipid peroxidation and accumulation of reactive oxygen species (ROS) in hepatocytes (Armiliato, 2015). ROS on the other hand play a vital role in the development of non-alcoholic fatty liver disease however the mechanism remains poorly understood (Ucar et al., 2013).

1.4.4 Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is reported to be the most common liver disease characterised by the excessive accumulation of triglycerides in the liver of people who consume little to no alcohol (Vernon et al., 2011; Yilmaz, 2012). NAFLD is a multifactorial disease with a variety of disease presentations such as hepatic steatosis, inflammation with steatosis and necrosis which eventually leads to cirrhosis (Lírio et al., 2016; Ragab et al., 2015). Studies have shown that the prevalence of NAFLD increases as the prevalence of obesity and metabolic syndrome increases (Armiliato, 2015; Jegatheesan and De Bandt, 2017). Approximately 33% of the population worldwide has been diagnosed with NAFLD

(Masarone et al., 2018). The prevalence is higher in the obese population whereby an estimated 75% has the disease (Masarone et al., 2018). The disease affects both children and adults and is closely linked with hepatic insulin resistance (Vos & Lavine, 2013). Several factors such as sex and age have effects on development of NAFLD; the increased consumption of fructose has been reported to be a major cause of the disease (Armiliato, 2015; Jegatheesan and De Bandt, 2017). Dietary fructose can directly cause NAFLD through direct hepatotoxic damage or indirectly by causing metabolic disorders which in turn increase the risk of developing NAFLD (Yilmaz, 2012). The overconsumption of fructose increases *de novo* lipogenesis (DNL) by activating key transcription factors such as carbohydrate-responsive element-binding protein (ChREBP) and sterol response element binding protein 1c (SREBP1c) (Jegatheesan & De Bandt, 2017). The resulting accumulation of lipids in the liver is usually followed by inflammation and damage to the liver (Armiliato, 2015). Thus this shows that there is a link between NAFLD and dyslipidaemia. Not only does insulin resistance cause NAFLD but it also causes dyslipidaemia.

1.4.5 Dyslipidaemia

Dyslipidaemia is an abnormal amount of lipids in the blood which is identified by increased free fatty acids, increased low-density lipoproteins (LDL), increased blood triglyceride (TG) levels and reduced high-density lipoproteins (HDL) (Lehtonen and Viikari, 1978). Dyslipidaemia plays a role in the development of cardiovascular diseases (Lehtonen and Viikari, 1978). Insulin resistance has been reported to decrease the ability of adipocytes to retain free fatty acids thus causing them to be released into the bloodstream (Kolovou et al., 2005). In the insulin resistant state, increased free fatty acid levels in turn cause an increase in TG levels by stimulating hepatic TG synthesis and promote the secretion of TG containing VLDL (Gorter et al., 2004). In most metabolic syndrome patients, decreased HDL levels are caused by increased TG levels (Martí, 2016). Increased TG levels cause the cholesteryl ester transfer protein to mediate the TG-cholesteryl ester exchange from VLDL to HDL particles thus forming TG-rich HDL particles (Martí, 2016). The TG-rich HDL particles are more prone to catabolism which thus leads to a decrease in HDL levels (Martí, 2016). LDL on the other hand is formed by components of small dense LDL particles (Packard, 2003) which is only noticeable when TG levels are increased (Kolovou et al., 2005). Increased TG levels perpetuate the accumulation of TG-rich VLDL molecules (Packard, 2003). Lipoprotein lipase causes lipolysis of TG-rich VLDL producing LDL particles with a different Apo protein B

conformation (Kolovou et al., 2005). The new LDL particles cannot bind to normal LDL receptors and thus accumulate in the bloodstream. Hepatic lipase then generates small dense LDL particles from new LDL particles which have been reported to play a major role in the development of cardiac diseases (Packard, 2003). Given the huge impact of obesity and its associated metabolic disorders on the healthcare sector, several treatment interventions have been implemented to manage these disorders.

1.5 Pharmacological agents for managing obesity and metabolic syndrome

Conventional pharmacological agents commonly used to treat obesity and its associated metabolic disorders have adverse effects (Bais et al., 2014). Thiazolinediones (TZDs) and biguanide metformin are mostly used to treat insulin resistance however both agents elicit undesirable side effects. For example, biguanide metformin can cause hypoglycaemia and physical weakness while TZDs can cause heart failure and weight gain (Tan et al., 2008). Other drugs commonly used are fibrates such Fenofibrate which forms the focus of the present study.

1.5.1 Fenofibrate (FF)

Fenofibrate (FF) is a fibric acid derivative (Noonan et al., 2013). FF is usually administered orally once daily due to its approximate half-life of 20 hours (Tsimihodimos et al., 2005). It is used to treat components of metabolic syndrome specifically hypertriglyceridemia or mixed hyperlipidaemia (Forcheron et al., 2002). This pharmacological agent is known to decrease total triglyceride and LDL concentration and to moderately increase HDL concentration (Forcheron et al., 2002). Upon administration, FF is rapidly metabolized into its active form of fibric acid, a peroxisome proliferator–activated receptor-α (PPARα) agonist which is mostly expressed in the liver (Tsimihodimos et al., 2005; Noonan et al., 2013). On activation of PPARα, free fatty acids are lowered through the upregulated synthesis of proteins which cause fatty acid transport and β-oxidation in metabolically active tissues (Noonan et al., 2013). These two processes result in an inhibition of the formation of VLDL and triglycerides (Noonan et al., 2013). Although FF has positive effects when treating metabolic disorders, studies have shown that it elicits serious side effects in growing children where chronic use leads to liver damage (Singh, 2006; Kalra et al., 2009). As it is the case with other conventional pharmacological agents, FF is expensive and in some communities, it is not

easily accessible hence the reliance on ethnomedicines in such communities (Mussarat et al., 2014).

1.6 Plant-derived ethnomedicines

Due to the cost of pharmacological agents, the use of medicinal plants is becoming popular in many societies. A recent study reported that approximately 80% of the population in developing countries rely on plants and commonly use them as traditional medicine (Mussarat et al., 2014). Plants, such as *Trigonella foenum-graecum*, have bioactive phytochemicals which possess properties of health benefit hence their popularity in traditional medicine (Bais et al., 2014; Ragab et al., 2015).

1.6.1 *Trigonella foenum-graecum*

Trigonella foenum-graecum L. (Fenugreek) is a commonly used medicinal plant. It belongs to the Leguminosae family. The plant is distributed across continents such as Africa, Europe, India, Mediterranean and Asia (Smith, 1973; Saxena and Vikram, 2004).

1.6.1.1 Botanical description

Fenugreek is an annually herbaceous and a diploid plant with a distinct taproot (Smith, 1973; Żuk-Gołaszewska and Wierzbowska, 2017). It mostly blossoms in June–July (Żuk-Gołaszewska and Wierzbowska, 2017) producing Papilionaceous flowers with an array of colours (cream, light purple and yellow) (Żuk-Gołaszewska and Wierzbowska, 2017). The leaves are three–lobed ovate leaflets (Żuk-Gołaszewska and Wierzbowska, 2017). The fenugreek plant produces horn-shaped pods which contain 10-20 seeds (Żuk-Gołaszewska and Wierzbowska, 2017). The seeds are small, flat and angular in shape and have four-faced stone-like structures that are gummy, sticky and fibrous in nature (Meghwal, 2012). The seeds contain an embryo which is bound by a semi-transparent endosperm (Żuk-Gołaszewska and Wierzbowska, 2017). The raw seeds are golden in colour and bitter in taste (Ahmad et al., 2016).



Figure 1.2: Fenugreek plant. (Source: Ghosh et al., 2015)

1.6.1.2 Medicinal uses

Fenugreek is most commonly consumed as a vegetable and a spice (Roberts, 2011). The plant is also an ethnomedicine for treatment of conditions such as inflammation, high cholesterol, visceral obesity, type 2 diabetes and dyslipidaemia (Lu et al., 2015; Meghwal, 2012; Żuk-Gołaszewska and Wierzbowska, 2017). Its other pharmacological properties include anti-hypertension, anti-tumor and anti-inflammatory (Chalaby, 2015; Mesallam et al., 2017). It can also be used as an antacid, laxative stimulant, anti-bacterial, anti-ulcerative, stomachic and anti-thrombotic agent (Chalaby, 2015; Mesallam et al., 2017). The most potent medicinal effects of fenugreek plant are found in the extract from its seeds hence, the seeds are considered to be the most valuable parts of the plant (Singh, Rai and Mahajan, 2016; Ahmad et al., 2016).

1.6.1.3 Chemical composition of seeds

The fenugreek seeds have a lipid content of approximately 100 g/kg. The fatty acid contents of fenugreek seeds (5-10%) are predominantly oleic, palmitic, linoleic and linolenic acid (Işikli and Karababa, 2005). The seeds contain carbohydrates (approximately 45-65%), flavonoids, saponins, choline, carotene, iron, zinc, calcium and phosphorus (Işikli and Karababa, 2005) as well as three bioactive compounds namely diosgenin, soluble fibre and 4-hydroxyisoleucine (Fuller and Stephens, 2015).

1.6.1.3.1 Diosgenin

Diosgenin is an active crystalline steroid saponin found abundantly in fenugreek seeds (Meghwal, 2012; Fuller and Stephens, 2015; Avalos-Soriano et al., 2016). Diosgenin in fenugreek seeds were reported to be hypocholesterolaemic as they form large micelles with bile salts thus increasing faecal excretion of bile salts and inhibiting cholesterol absorption which ultimately decreases cholesterol concentrations (Avalos-Soriano et al., 2016; Kumar et al., 2015). Furthermore, diosgenin was reported to decrease the serum triglycerides and LDL (Avalos-Soriano et al., 2016; Żuk-Gołaszewska and Wierzbowska, 2017). It has also been reported to contribute to the maintenance of insulin signalling and glucose homeostasis by restoring pancreatic β-cells and downregulating enzymes involved in glucose export and hepatic gluconeogenesis (Fuller and Stephens, 2015).

1.6.1.3.2 Soluble fibre

Fenugreek seeds are also rich in fibre which amount to approximately 50% of the dry seeds (20% insoluble fibre and 30% soluble fibre) (Fuller and Stephens, 2015). The fibre in Fenugreek seeds are mainly galactomannans which has medicinal properties to regulate glucose metabolism (Ahmad et al., 2016; Fuller and Stephens, 2015). The soluble and insoluble fibre regulate glucose metabolism by slowing down postprandial glucose absorption which is responsible for hypoglycaemic effects of the seeds (Avalos-Soriano et al., 2016).

1.6.1.3.3 The amino acid derivative 4-Hydroxyisoleucine

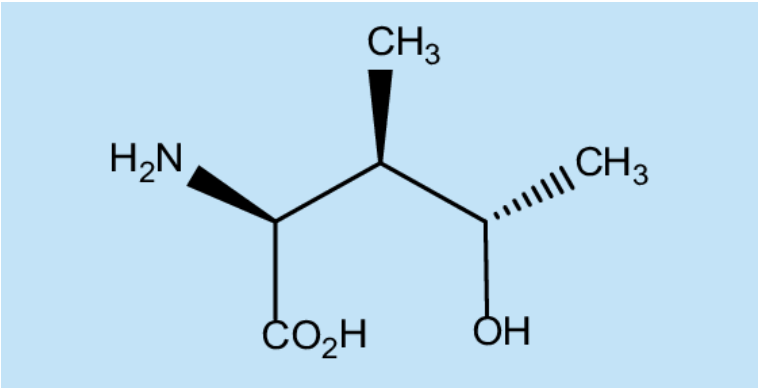


Figure 1.3: The chemical structure of 4-hydroxyisoleucine. (Source: Patel, 2014)

4-Hydroxyisoleucine (4-OH-Ile) is a novel branched-chain amino acid derivative (Fuller and Stephens, 2015). It is a major compound found in fenugreek plant and it is the major compound that is responsible for physiological functions of the seeds (Jaiswal et al., 2012; Shi et al., 2017). The seeds have approximately 30-50% of total amino acid content of which 4-OH-Ile makes up 80% of total amino acid content (Smith, 1973; Nagulapalli Venkata et al., 2017). 4-OH-Ile has functional properties such as antidiabetic and anti-hyperlipidaemic as glucose and lipid metabolisms have been shown to be linked (Zafar and Gao, 2016).

1.6.1.3.3.1 Antidiabetic properties of 4-OH-Ile

4-OH-Ile has been reported to cause glucose-induced insulin release in vivo and in vitro (Saxena and Vikram, 2004). 4-OH-Ile stimulates the release of insulin only in the presence of elevated blood glucose concentrations in the range of 8.3 mM - 16.7 mM (Avalos-Soriano et al., 2016). In addition, 4-OH-Ile improves insulin resistance by enhancing islet β-cell insulin secretion (Lu et al., 2015). Furthermore, 4-OH-Ile was reported to increase peripheral glucose uptake and reduce hepatic glucose output (Roberts, 2011) as well as enhance the translocation of GLUT4 to the cell surface through a phosphatidylinositol-3-kinase (PI-3-K)/AKT-dependent pathway which thus enhances the uptake of glucose in skeletal muscle cells (Jaiswal et al., 2012).

1.6.1.3.3.2 Anti-hyperlipidemic properties of 4-OH-Ile

Research has shown that 4-OH-Ile administered orally at 50 mg/kg body weight decreased total cholesterol concentrations, plasma triglycerides and increased HDL cholesterol concentrations of rats (Roberts, 2011). The administration of 4-OH-Ile to diabetic rats also caused an increase in HDL (Zafar and Gao, 2016).

1.7 Rationale

Although a number of studies have evaluated the medicinal potential of fenugreek, these studies were conducted on adult male rats with chemically induced metabolic diseases (Mohamed et al., 2015; Pradeep and Srinivasan, 2018; Mukthamba and Srinivasan, 2017). These models however, do not mimic the disease pathogenesis in young children on high fructose diets. Thus, a study using growing animal models to mimic growing children will be

of benefit. Previous studies mostly utilized only male rats (Rawat et al., 2014; Mohamed et al., 2015) but studies have shown the existence of sexually dimorphic responses to therapeutic interventions (K G Ibrahim et al., 2019). Thus there is a need to evaluate the effect of 4-OH-Ile in both sexes under similar experimental design. Furthermore, most studies also utilized the crude extract or powdered form of fenugreek seeds instead of the compound of interest i.e. 4-OH-Ile (Kumar et al., 2014). In order to fully characterize the medicinal potential of plants, it is important to validate efficacy of individual phytochemicals so as to develop a pure bioactive medicine. Fenugreek has three bioactive compounds (diosgenin, soluble dietary fiber and 4-OH-Ile) but this study focused on medicinal properties of 4-OH-Ile of which little is known about its effects (Wang & Rosett, 2008).

1.8 Aim

The study aimed to investigate whether orally administered 4-OH-Ile can prevent the development of high-fructose diet-induced metabolic dysfunction in growing male and female *Sprague Dawley* rats.

1.9 Objectives

The specific objectives of the study were to investigate the potential protective effects of 4-OH-Ile in high-fructose fed growing rats on:

i. General metabolic dysfunction as measured by
   - Visceral adiposity
   - Serum hormones associated with obesity (insulin and adiponectin)
   - Circulating metabolic substrates (glucose, cholesterol and triglycerides)
   - Haematocrit and haemoglobin concentrations

ii. The development of non-alcoholic fatty liver disease (NAFLD) as measured by
   - Hepatic histology scores for NAFLD
   - Development of fibrosis

iii. Kidney function as measured by
   - Kidney histology analysis and development of fibrosis

1.10 Hypothesis

H_1:4-Hydroxyisoleucine (4- OH-Ile) prevents the development of high-fructose-diet-induced metabolic dysfunction in growing *Sprague Dawley* rats.

H_0: 4-Hydroxyisoleucine (4- OH-Ile) does not prevent the development of high-fructose-diet-induced metabolic dysfunction in growing *Sprague Dawley* rats.

CHAPTER 2: MATERIALS AND METHODS

2.1 Source of 4-hydroxyisoleucine

The 4-OH-Ile used in this study was a white crystalline powder purchased from Chempure (Catalogue no: Y1712019; Pretoria, South Africa) with a purity of 98.5%. The certificate of analysis was provided by Suzhou Vitajoy Biotech (China).

2.2 Ethical clearance for animal use

The study was approved by the Animal Research Ethics Committee (AESC: 2018/03/14B) of the University of the Witwatersrand, Johannesburg, South Africa. A copy of the ethics clearance certificate (including approval for modifications and extensions) is included as appendix 1.

2.3 Animal housing and general care

Twenty-one-day old weanling male (n = 40) and female (n = 40) *Sprague Dawley* rat pups, were supplied by and housed at the Central Animal Services (CAS) Unit of the University of the Witwatersrand. Different rat types respond differently to experimental procedures (Rex et al., 2004). *Sprague Dawley* rats are used mostly in laboratory experiments as they mimic human pathologies adequately compared to other rat types. The Sprague Dawley strain is commonly used in metabolic studies (Kasimu G. Ibrahim et al., 2019). The rat pups were individually housed in Perspex cages lined with clean wood shavings as bedding. The wood shavings were changed once a week. The room temperature was maintained at 25 ± 2 ºC and a 12-hour light/dark cycle was followed with the lights on between 7 am and 7 pm. The rats had *ad libitum* access to commercial standard rat chow (crude protein 240 g/kg, crude fibre 45 g/kg, crude oils and fats 53 g/kg, calcium 14 g/kg and phosphorous 8 g/kg) (LabChef Nutritionhub, Stellenbosch, South Africa) and to drinking fluids which were either plain drinking water or 20% fructose solution.

2.4 Treatment doses and preparation of drug vehicle

Gelatine cubes were used as vehicle media to administer 4-OH-Ile or the Fenofibrate (FF). The cubes were prepared as described by Kamerman et al. (2004) but with modifications whereby sugar and Bovril content were half of that used by Kamerman et al (2004). The cubes contained: 8 g of brown sugar (Selati, RCL Foods (Pty) Ltd, South Africa), 8 g of Sheridans gelatine (Libstar, Operations (Pty) Ltd, South Africa) and 2.5 g of Beefy Bovril

(Pioneer Foods, South Africa) mixed with 100 ml of water at 55-60 °C. The solution containing either 4-OH-Ile, FF or no drug (gelatine mixture only) was drawn up into a syringe and then placed into ice cube trays and then allowed to cool down to form 1 ml gelatinous cubes. 4-OH-Ile was used at a dose of 500 mg/kg body weight per day in this study which was similar to the dose used by Sharma and Choudhary (2014) while comparing the effectiveness of fenugreek seeds and atorvastatin on experimentally induced hyperlipidaemia. In addition, FF was used at a dose of 100 mg/kg body weight per day similar to the dose used by Oidor-Chan et al. (2016) while investigating the cardio-protective effect of a combination of FF and Metformin in Type 2 diabetes and an acute myocardial infarction model.

2.5 Drinking fluid preparation

20% Fructose solution was prepared by dissolving 800 g of GMO free and bio-friendly fructose granulated fruit sugar (energy 1680 kJ, protein 0 g, glycaemic carbohydrate 98.2 g, fat 0 g, fibre 0 g and total sodium 12 mg) (Nature's Choice fructose, South Africa) in 4 L of water at 55-60 °C. After the fructose solution was cooled to room temperature, a drop of red food colouring (Robertsons, Retailer Brands (Pty) Ltd, South Africa) was added for identification purposes. To distinguish the plain drinking water from the red–coloured fructose solution, a drop of blue food colouring (Robertsons, Retailer Brands (Pty) Ltd, South Africa) was added to 5 L of tap water.

2.6 Experimental groupings and treatments

Eighty 21-day old male and female *Sprague Dawley* rats were habituated (put in individual cages and fed standard rat chow, plain gelatine cubes and plain drinking water) for two consecutive days before they were randomly allocated to five experimental groups of 16 pups each (i.e. males: n = 8; females: n = 8). The random allocation was done by blindly taking rats from a cage and then allocating them to randomly to a group based on the sex of the rat. The treatment groups were as follows: group 1 (negative control) received plain gelatine cubes (PGC) and plain drinking water (PDW); group 2 (in which metabolic dysfunction was induced) received 20% fructose solution (20% FS) (Sandeva et al., 2015) and PGC; group 3 (in which the prophylactic effects of 4-OH-Ile were investigated) received 20% FS and 500 mg/kg body weight per day of 4-OH-Ile administered in gelatine cubes (4HGC); group 4 (in which the effects of 4-OH-Ile were investigated in the absence of fructose feeding) received PDW and 4HGC per day and group 5 (positive control) received 20% FS + FF (100 mg/kg

body weight per day) (Oidor-Chan et al., 2016) in gelatine cubes (FENGC). All the rats in each experimental group underwent treatment for 10 weeks consecutively. There were no morbidities or mortalities recorded in the study. The experimental design is summarised in figure 2.1.

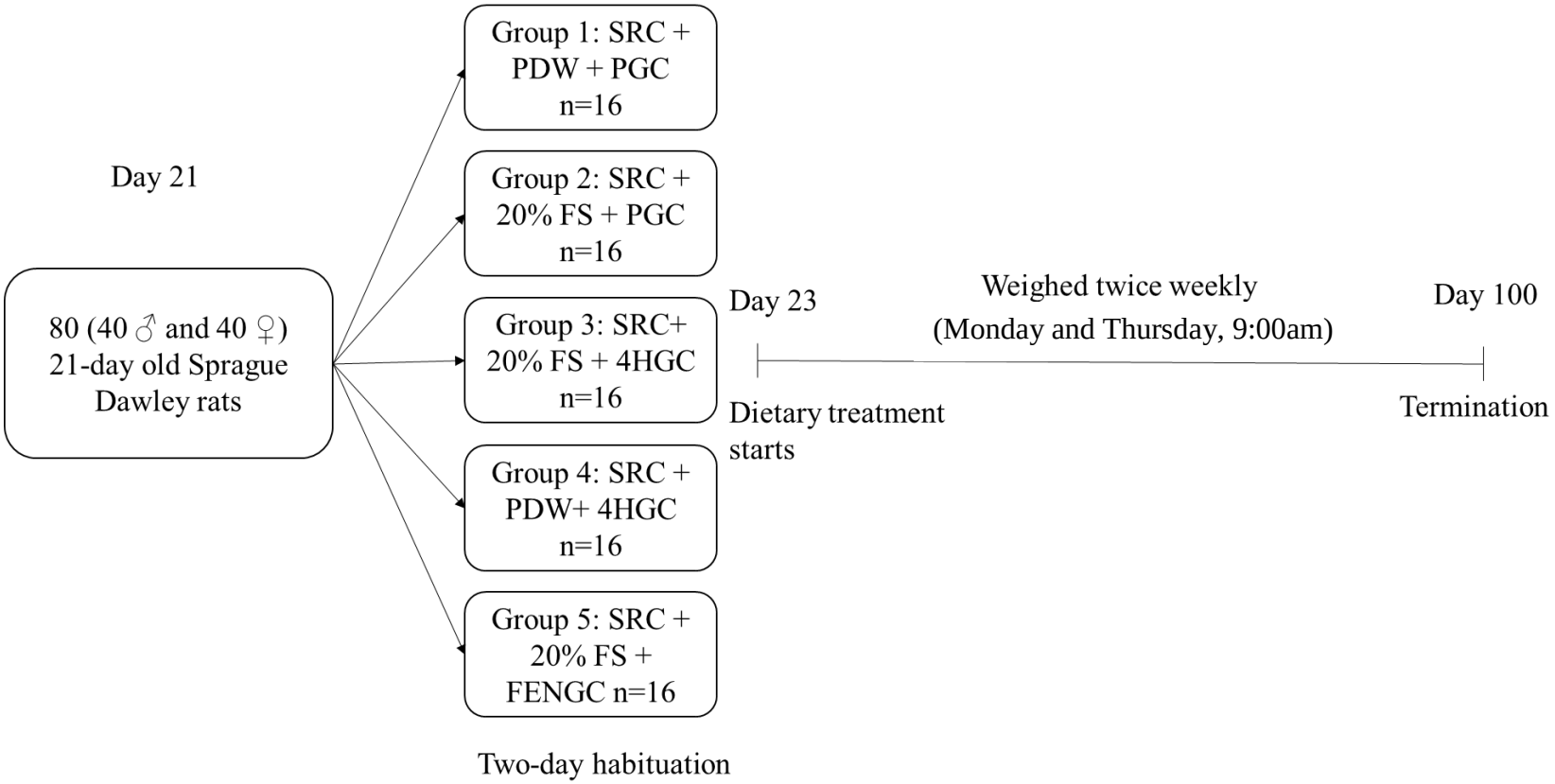


Figure 2. 1: A summary of the experimental design and groupings showing the sample size and the treatment plans.

SRC – standard rat chow, PDW – plain drinking water, PGC – plain gelatine cube, 4HGC – 500 mg/kg body weight of 4-hydroxyisoleucine (4-OH-Ile) in a gelatine cube, FENGC – 100 mg/kg body weight of Fenofibrate in a gelatine cube, 20%FS – 20% fructose solution.

2.7 Measurements, experimental interventions and terminal procedures

2.7.1 Body weight

The rats were weighed individually on induction with an electronic balance (Snowrex Electronic Scale, Clover Scales, Johannesburg). Thereafter, the rats were weighed twice a week over a 10–week experimental period in order to monitor the health of the animals, the growth rate and to maintain accurate daily dose of 4-OH-Ile or Fenofibrate (Bais et al., 2014).

For each rat, total weight gain percentage was determined as follows:

Weight gain (g) = Terminal weight – induction weight

Weight gain percentage (%) = (Weight gain / induction weight) × 100

2.7.2 Terminal procedures

At the end of the 10–week experimental period, the rats were fasted overnight for approximately 8 hours. Thereafter, terminal body weights were determined with an electronic balance (Snowrex Electronic Scale, Clover Scales, Johannesburg). The tail vein of the rats was pricked with a needle; oozed blood was used for determination of the fasting blood glucose concentrations using a calibrated glucometer (Ascensia EliteTM Blood glucose meter, Mishawaka, USA). A drop of blood was used to determine haematocrit and haemoglobin concentrations using a haematocrit meter (InSight HCT Meter, Woodley Equipment Company Ltd, Bolton BL6 7NY, UK). The rats were then euthanized by an intraperitoneal injection of sodium pentobarbitone administered at an overdose of 200 mg/kg body weight (Eutha-naze, Centaur labs, Bayer, Johannesburg, South Africa).

2.7.2.1 Blood collection, processing and storage of plasma

A midline incision was made to expose the heart; blood was then collected into heparinized blood tubes via cardiac puncture using 10 ml syringes and 20 G needles. The collected blood was centrifuged (Sorvall RT 6000B, Pegasus Scientific Inc, Rockville USA) for 15 min at 5000 RPM at 20°C. The blood plasma was collected in microtubes and stored at -20 °C for subsequent determination of insulin, adiponectin, cholesterol and triglyceride concentrations.

2.7.2.2 Determination of visceral organ morphometry

Following cardiac puncture, visceral fat, liver and kidneys were carefully dissected and weighed (Presica balance, Insrulab, Switzerland). After weighing the liver and kidneys, a sample of the left median lobe of the liver and either one of the kidneys were preserved in 10% phosphate buffered formalin for histological analysis. Other visceral organs (heart, pancreas, small and large intestine, caecum and stomach) were dissected and also weighed.

2.7.2.3 Determination of indexes of long bone growth

The right hind leg of the rat carcass was detached from the pelvis and de-fleshed. Then the femur and tibia were disarticulated and removed. The de-fleshed femur and tibia were subsequently dried in an oven (Salvis ®, Salvis Lab, Switzerland) at 40 ºC for 7 days. Thereafter, the mass of the femur and tibia was determined using an electronic balance (Presica balance, Insrulab, Switzerland) and length was measured using a Digital Vernier calliper (Mastercraft tools, South Africa). The bone densities of the femur and tibia were calculated as follows:

Bone density = bone mass (mg)/bone length (mm) (Seedor et el. 1991).

2.7.2.4 Determination of plasma insulin concentration

An enzyme linked immunosorbent assay (ELISA), specifically the rat insulin kit (Elabscience®, Rat Insulin ELISA Kit, Hubei, China), was used to determine the plasma insulin concentrations according to the manufacturer's instructions. A quantitative sandwich enzyme immunoassay technique which uses a monoclonal antibody specific for rat insulin was used in the assay. Briefly, 100 µL of plasma sample was added to each well and incubated for 90 min at 37 ºC. The liquid was then removed from the well and 100 µL of Biotinylated Detection antibody was added and the plate incubated for 60 min at 37 ºC. The plate was washed three times before 100 µL HRP Conjugate was added and then incubated for 30 min at 37 ºC. Thereafter, the well was washed five times before 90 µL substrate reagents was added to the well and then incubated for 15 min at 37 ºC. After the incubation, 50 µL stop solution was added to the well. The absorbance was read at 450 nm using a plate reader (Multiskan Ascent, Lab system, model nº 354, Helsinki, Finland). A standard curve was developed using calibrator concentration from which the insulin concentrations (ng/mL) in the samples were determined.

2.7.2.5 Computation of the Homeostatic model of insulin resistance (HOMA IR) index

A HOMA-IR index was used to analyse insulin resistance (Matthews et al., 1985) and was computed using the equation:

$$\text{HOMA-IR} = [\text{fasting insulin } (\mu U/mL) \times \text{fasting glucose (mmol/ L)}] / 22.5.$$

A conversion factor of 0.02 was used to convert insulin concentration from ng/mL to μU/mL

2.7.2.6 Determination of plasma adiponectin concentration

An ELISA, specifically the rat adiponectin kit (Elabscience®, Rat Adiponectin ELISA Kit, Hubei, China), was used to determine plasma adiponectin concentrations according to the manufacturer's instructions. A quantitative sandwich enzyme immunoassay technique which uses a monoclonal antibody specific for rat adiponectin was used in the assay. 100 μL plasma samples was added to each well and then incubated for 90 min at 37 ºC. The liquid was removed before 100 μL Biotinylated Detection antibody was added and then incubated for 60 min at 37 ºC. Subsequently, the plate was washed three times, and then 100 μL HRP Conjugate was added before the plate was incubated for another 30 min at 37 ºC. The plate was washed five times and then 90 μL Substrate Reagent was added before the plate was incubated again for 15 min at 37 ºC. Thereafter, 50 μL stop solution was added before the absorbance was read at 450 nm using a plate reader (Multiskan Ascent, Lab system, model nº 354, Helsinki, Finland). A standard curve was developed using calibrator concentration from which the adiponectin concentrations (pg/mL) in the samples were determined.

2.7.2.7 Determination of plasma lipid (cholesterol and triglyceride) concentrations

A Colorimetric method was used to determine both plasma cholesterol and triglyceride concentrations using a Total Cholesterol Assay Kit (Elabscience®, Total cholesterol assay kit, Hubei, China) and a Triglyceride Assay Kit (Elabscience®, Triglyceride assay kit, Hubei, China) respectively. 1000 μL of the working solution was added to 10 μL of plasma sample in a glass test tube. The test tubes were then incubated for 10 min at 37 ºC. Then 3 ml of solution from each test tube was poured into a 4 ml cuvette and the absorbance was read at 510 nm using a spectrophotometer (Beckman Coulter DU ®720 General Purpose UV/Vis, USA). The spectrophotometer was set to zero with distilled water in order to establish the blank reading.

The unit for the concentration or level of absorbance of the samples, blank and standard can also be recorded as optical density (OD).

a. The cholesterol concentrations (mmol/L) were determined using the following equation:

   $T_{cholesterol} = [(OD_{sample} - OD_{blank}) / (OD_{standard} - OD_{blank})] \times std_{concentration}$

   $T_{cholesterol}$ = total cholesterol concentration

   OD = optical density

   Std= standard

b. The triglyceride concentrations (mmol/L) were determined using the following equation:

   $T_{triglyceride} = [(OD_{sample} - OD_{blank}) / (OD_{standard} - OD_{blank})] \times std_{concentration}$

   $T_{triglyceride}$ = total triglyceride concentration

   Std = standard

   OD = optical density

2.7.2.8 Histology for determination of non-alcoholic fatty liver disease and hepatic fibrosis

The fixed liver samples were processed for haematoxylin and eosin (H & E) staining to detect any hepatocellular changes or Masson's trichrome (MT) to reveal the presence of fibrotic tissue in the liver using an automated tissue processing machine (Microme STP 120, Thermo Fisher Scientific, Walldorf, Germany). The tissues were dehydrated in graded alcohol concentrations, briefly cleared in toluene before immersing in melted paraffin. The tissue samples were then embedded in paraffin wax in wax blocks (Thermolyne-Histo-Center HC33220-26, Barnstead, Dubuque, Iona, USA) before sectioning at 5 μm with a microtome (Leica Biosystems RM2125 RTS, China). Subsequently, sections on glass slides were deparaffinised with xylene, then hydrated in graded alcohol concentrations before staining with H & E or MT. After staining, sections were once again dehydrated and cleared, cover slipped with a mounting agent before being viewed under a light microscope (Olympus Bh2, Japan).

2.7.2.8.1 NAFLD Activity Score

The degree of NAFLD was determined by using NAFLD Activity Score (NAS) criteria as described by Liang et al. (2014). According to these criteria, macrovesicular and microvesicular steatosis (accumulation of fat droplets), inflammation (aggregation of Kupffer cells) and hypertrophy (increase in size of fat cells) of the hepatocytes are scored by observing the affected area at 40 times objective. The microvesicular, macrovesicular steatosis and hypertrophy were scored such that a score of 0 = <5%, 1 = 5–33%, 2 = 33–66%, and 3 = >66% represent the area affected. Inflammation was graded such that a score of 0 = no Kupffer cells aggregated, 1 = 0.5–1 Kupffer cells aggregated, 2 = 1– 2 Kupffer cells aggregated, 3 = >2 Kupffer cells aggregated while observing at 40 times objective.

2.7.2.8.2 Hepatic fibrosis

The fibrosis in the liver was analysed from images taken at 100 times objective using ImageJ software (ImageJ 1.47v/Java 1.6.0_045, Oracle America Inc.) (Schneider et al., 2012). The area per point (A_p) and area fraction ($A_{fraction}$) of each liver section consisting of connective tissue were determined by using the point count method to obtain the sum of points ($\sum_p$). A total of 5 random camera fields (stage moved linearly every 2 mm) were used for each section. The formula used to determine the area per point was (Ibrahim et al., 2019):

$A_p = A \div \sum_p$

A_p = area per point

A = area

$\sum_p$ = sum of points falling on connective tissue within a camera field of an area of 7339.52 μm²

The formula used to determine the area fraction of an image with the area size of 594501.7232 μm² was (Ibrahim et al., 2019):

$A_{fraction} = [(A \div 594501.7232\ \mu m^2) \times 100]$

$A_{fraction}$ = area fraction

A = area

2.7.2.9 Assessment of kidney histopathology and renal fibrosis

The fixed kidney samples were processed for H & E staining to detect any alterations to kidney morphology or MT to reveal the presence of fibrotic tissue in the kidney using an automated tissue processing machine (Microme STP 120, Thermo Fisher Scientific, Walldorf, Germany). The tissues were dehydrated in graded alcohol concentrations, briefly cleared in toluene before immersing in melted paraffin. The tissue samples were then embedded in paraffin wax using wax blocks (Thermolyne-Histo-Center HC33220-26, Barnstead, Dubuque, Iona, USA) before sectioning at 5μm with a microtome (Leica Biosystems RM2125 RTS, China). Subsequently, sections on glass slides were deparaffinised with xylene, then hydrated in graded alcohol concentrations before staining with H & E or MT. After the staining, sections were once again dehydrated and cleared, cover slipped with a mounting agent before being viewed under a light microscope (Olympus Bh2, Japan).

2.7.2.9.1 Kidney morphology

Alterations to kidney morphology was analysed by measuring renal corpuscle area (A_R) and glomerulus area (A_G) using ImageJ software (ImageJ 1.47v/Java 1.6.0_045, Oracle America Inc.) from photomicrographs taken at 40 times objective. From these measurements, area of Bowman's capsule space (A_B) was calculated [area of renal corpuscle (A_R) - area of glomerulus (A_G)].

2.7.2.9.2 Renal fibrosis

The development of fibrosis in the kidneys was quantitatively assessed using the MT-stained slides at 40 times objective using ImageJ software (ImageJ 1.47v/Java 1.6.0_045, Oracle America Inc.) (Schneider et al., 2012). The area per point (A_p) and area fraction ($A_{fraction}$) of each kidney section consisting of connective tissue at 10 times objective were determined by using the point count method to obtain the sum of points ($\sum_p$). A total of 5 camera fields (stage moved linearly every 2 mm) were used for each section. The formula used to determine the area per point was (Ibrahim et al., 2019):

$A_p = A \div \sum_p$

A_p = area per point

A = area

$\sum_p$ = sum of points falling on connective tissue within a camera field of an area of 7339.52 μm²

The formula used to determine area fraction of an image with the area size of 594501.7232 μm² was (Ibrahim et al., 2019):

$A_{fraction} = [(A \div 594501.7232\ \mu m^2) \times 100]$

$A_{fraction}$ = area fraction

A = area

The formula used to determine area of the Bowman's capsule space was:

$A_B = A_R - A_G$

A_B = area of the Bowman's capsule space

A_R = area of renal corpuscle

A_G = area of glomerulus

2.8 Statistical analysis

Graphpad Prism version 7 statistical software (Graph-pad Software Inc., San Diego, USA) was used to analyse data. Parametric data were expressed as mean and standard deviation. Non-parametric data were expressed as median and interquartile range. For all statistical analyses, the level of statistical significance was set at $p < 0.05$. A one-way ANOVA was used to analyse multi-group parametric data whilst a Kruskal – Wallis test was used to analyse non-parametric data. Where the differences in mean or median was significant, a Bonferroni *post hoc* test (for parametric data) or a Dunn's *post hoc* test (for non-parametric data) was used.

CHAPTER 3: RESULTS

3.1 Growth performance of the rats

The results of growth performance include the body weights, body weight gain percentages and the linear bone growth measurements for the female and male rats.

3.1.1 Absolute body weight and percentage body weight gain

The body weight (induction and terminal) and body weight gain percentage of the female rats across the different dietary treatment groups are shown in Figures 3.1 and 3.2 respectively. There was no significant difference in induction body weight ($p=0.97$) as well as in the terminal body weight ($p=0.92$) of the female rats across the different treatment groups. All rats grew significantly ($p=0.0001$) over the 10 week experimental period. There was also no significant difference in overall weight gain percentage ($p=0.86$) of the female rats across the dietary treatment groups.

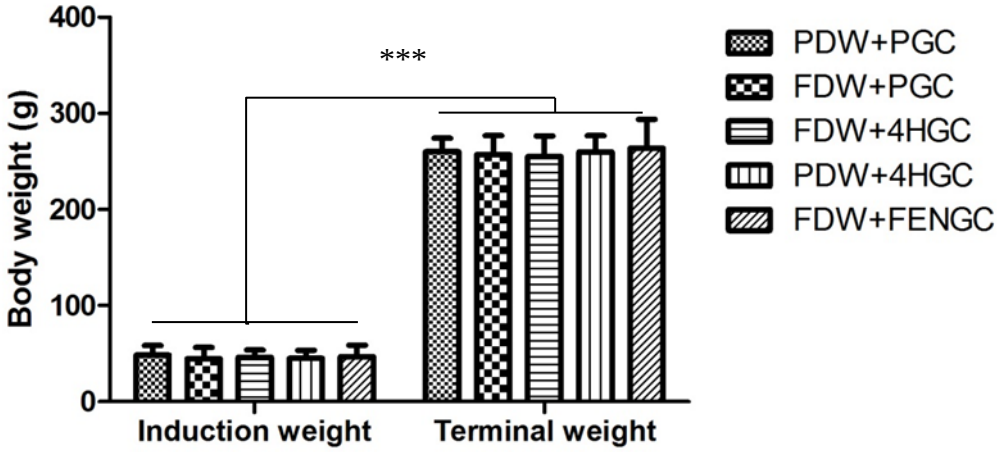


Figure 3. 1: The induction and terminal weights of female rats across different dietary treatment groups.

Data is presented as mean ± SD; n=8 females per dietary group. *** $p < 0.0001$. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

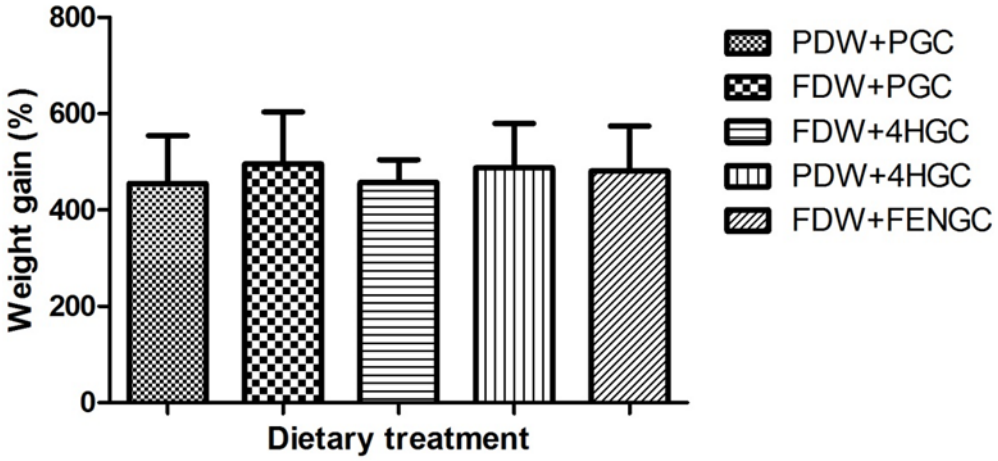


Figure 3. 2: The overall weight gain percentage of the female rats across different dietary treatment groups.

Data is presented as mean ± SD; n=8 females per dietary group. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

The body weight (induction and terminal) and body weight gain percentage of the male rats across the different dietary treatment groups are shown in Figures 3.3 and 3.4 respectively. There was no significant difference in induction body weight ($p=0.34$). The terminal body weight of the male rats was also not significantly different ($p=0.15$) across the groups except for those administered fructose and 4-OH-Ile (FDW+4HGC) which had a significantly lower terminal body weight ($p=0.01$) compared to the males administered the normal diet (PDW+PGC). Similar to the female group, all the male rats grew significantly ($p=0.0001$) after the 10 week experimental period. The overall percentage weight gain of the male rats across the different dietary groups was also not significantly different ($p=0.82$).

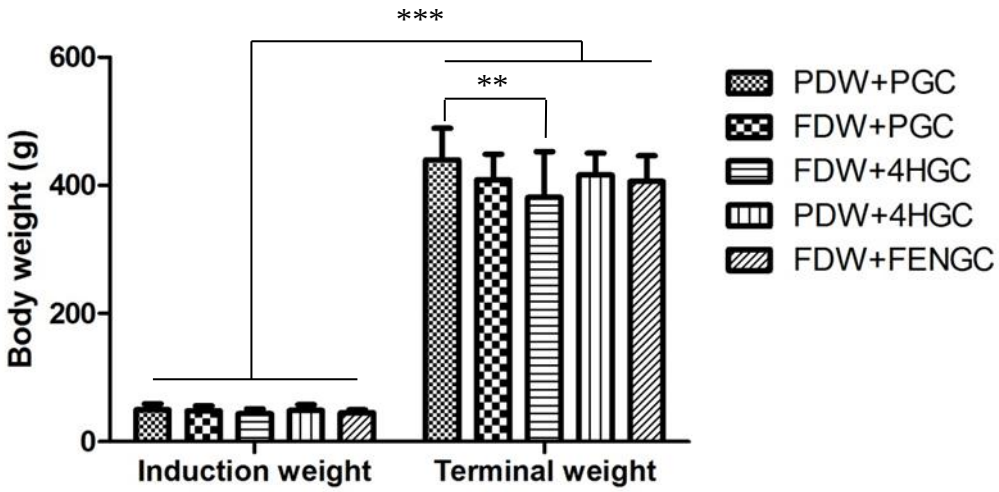


Figure 3. 3: The induction and terminal weights of male rats across different dietary treatment groups.

Data is presented as mean ± SD; n=8 males per dietary group. *** p< 0.0001, **p<0.01. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

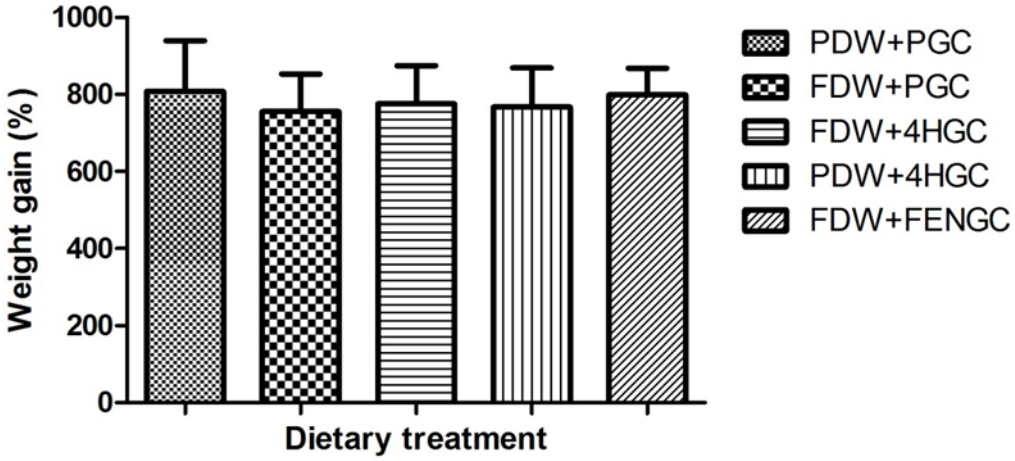


Figure 3. 4: The overall weight gain percentage of the male rats across different dietary treatment groups.

Data is presented as mean ± SD; n=8 males per dietary group. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

3.1.2 Long bone growth parameters

The length (mm), masses (mg) and relative densities (mg/mm) of the tibia and femora of the female rats across the different dietary treatment groups over the 10-week feeding period are illustrated in Table 3.1. For tibia bone measurement, there was no significant difference in the length ($p=0.07$), mass ($p=0.78$) and relative density ($p=0.96$) of the female rats across the different dietary groups. There was similarly no significant difference ($p=0.05$) in the length ($p=0.22$), mass ($p=0.81$) and densities ($p=0.44$) of the femur bone for the female rats. Representative radiographs highlighting the aforementioned parameters of the tibia and femora of the female rats for the different dietary groups are shown in Figure 3.5.

Table 3. 1: Effects of 4-hydroxyisoleucine on the tibia and femora length, mass and density of the female rats across different dietary treatment groups after 10 weeks

	PDW + PGC	FDW + PGC	FDW + 4HGC	PDW + 4HGC	FDW + FENGC
Tibia					
Length (mm)	39.45±1.84 [a]	38.18±1.17 [a]	37.50±0.49 [a]	37.32±2.37 [a]	37.39±1.34 [a]
Mass (mg)	478.10 ±38.22 [a]	468.30 ±51.78 [a]	450.30 ±45.72 [a]	459.80 ±90.33 [a]	443.50 [a]±44.17 [a]
Density (mg/mm)	12.12 ±0.74 [a]	12.26 ±1.35 [a]	12.00 ±1.14 [a]	12.31 ±2.15 [a]	11.84 ±0.88 [a]
Femur					
Length (mm)	33.03 ±0.61 [a]	32.19 ±1.03 [a]	32.85 ±0.58 [a]	33.68 ±2.18 [a]	32.68 ±0.85 [a]
Mass (mg)	593.40 ±38.48 [a]	572.30 ±57.38 [a]	591.80 ±53.25 [a]	568.80 ±69.77 [a]	570.60 ±47.65 [a]
Density (mg/mm)	17.96 ±1.02 [a]	17.76 ±1.43 [a]	18.01 ±1.51 [a]	16.87±1.57 [a]	17.45±1.18 [a]

Data is presented as mean ± SD; n=8 females per dietary group. [a] Within row means with similar superscripts are not significantly different at $p>0.05$. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

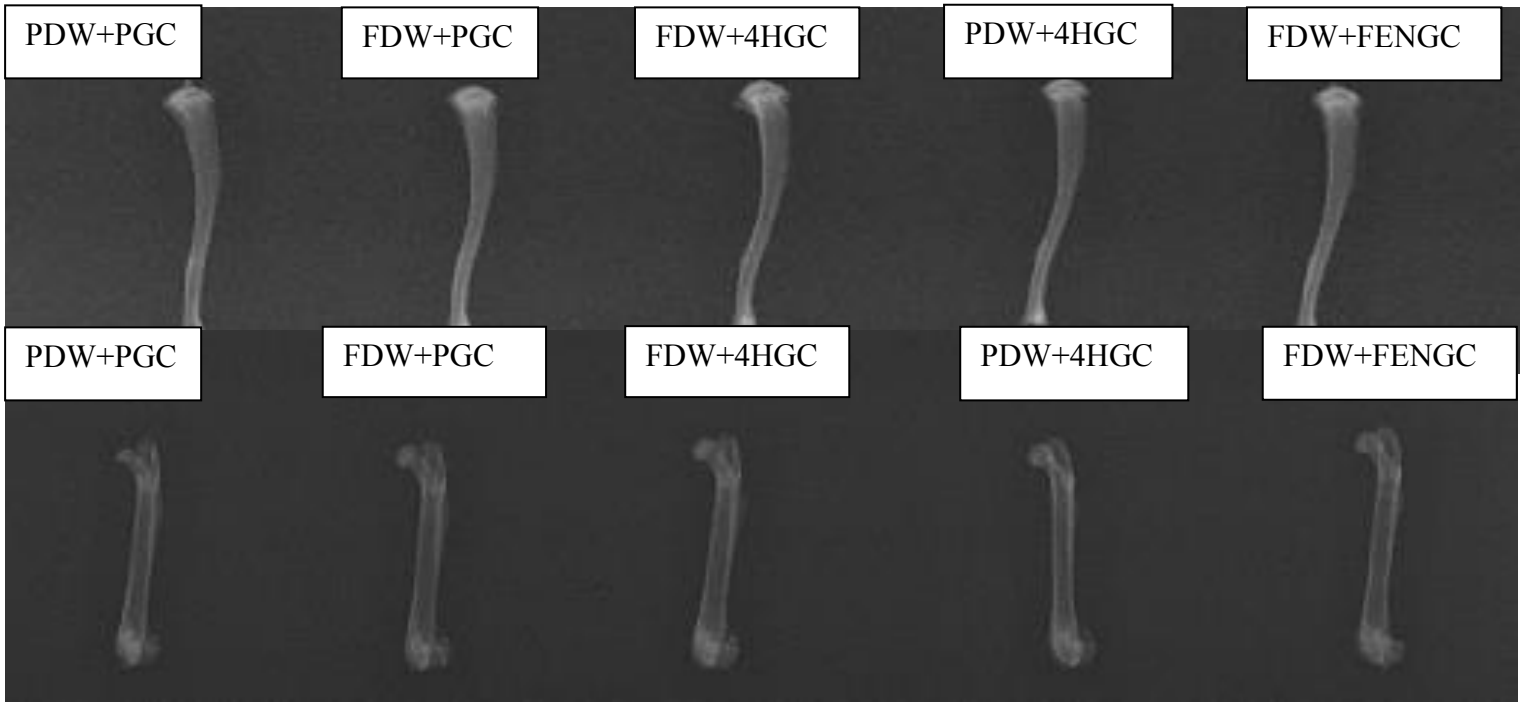


Figure 3. 5: Representative radiograph images of the tibiae and femora of the female rats.

The top row shows radiographs of the tibiae and the bottom row shows radiographs of the femora of the female rats from each of the groups. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

The length (mm), masses (mg) and relative densities (mg/mm) of the tibia and femora of the male rats across the different dietary treatment groups over the 10-week feeding period are illustrated in Table 3.2. For tibia bone measurement, there was no significant difference in the length ($p=0.52$), mass ($p=0.32$) and relative density ($p=0.39$) of the male rats across the different dietary groups. There was similarly no significant difference in the length ($p=0.14$), mass ($p=0.12$) and densities ($p=0.23$) of the femur bone for the male rats. Representative radiographs of the tibia and femora of the male rats for the different dietary groups are shown in Figure 3.6.

Table 3. 2: Effects of 4-hydroxyisoleucine on the tibia and femora length, mass and density of the male rats across different dietary treatment groups after 10 weeks

	PDW + PGC	FDW + PGC	FDW + 4HGC	PDW + 4HGC	FDW + FENGC
Tibia					
Length (mm)	41.70±2.52[a]	41.52±1.915[a]	40.49±2.93[a]	42.42±1.28[a]	40.87±2.13[a]
Mass (mg)	621.40±78.08[a]	580.10±70.30[a]	537.90±96.47[a]	611.60±83.00[a]	591.70±84.93[a]
Density (mg/mm)	14.88±1.36[a]	13.94±1.25[a]	13.29±2.15[a]	14.40±1.79[a]	14.43±1.56[a]
Femur					
Length (mm)	37.57±1.20[a]	35.79±1.07[a]	35.90±2.47[a]	36.59±1.23[a]	36.18±0.10[a]
Mass (mg)	790.30±84.64[a]	721.90±65.46[a]	664.70±128.10[a]	756.30±86.31[a]	746.80±44.51[a]
Density (mg/mm)	21.04±2.22[a]	20.14±1.36[a]	18.49±3.12[a]	20.64±1.95[a]	20.64±1.19[a]

Data is presented as mean ± SD; n=8 males per dietary group. [a] Within row means with similar superscripts are not significantly different at $p>0.05$. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

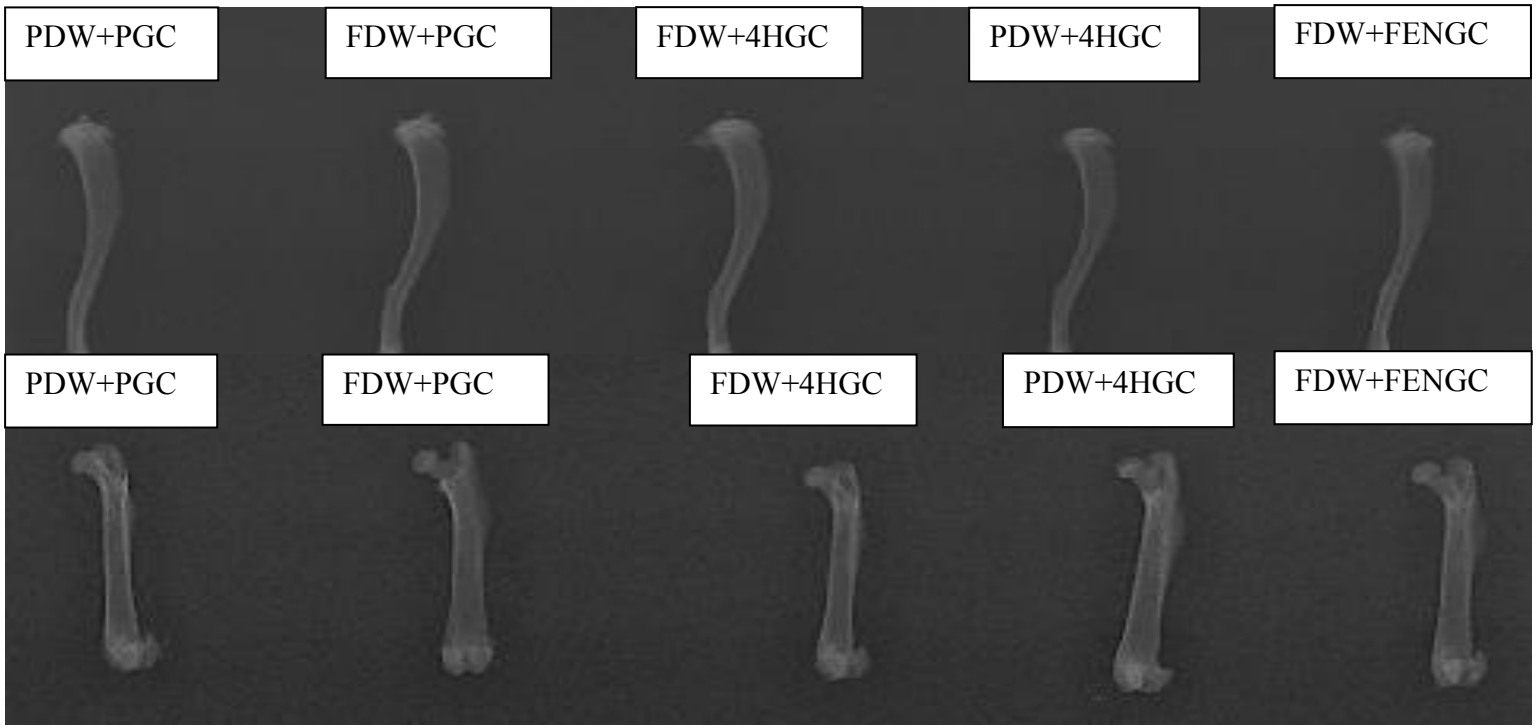


Figure 3. 6: Representative radiograph images of the tibiae and femora of the male rats.

The top row shows radiographs of the tibiae and the bottom row shows radiographs of the femora of the male rats from each of the groups. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

3.2 The red blood cells parameters (haemoglobin and haematocrit)

The haemoglobin concentrations and haematocrit of the female rats are illustrated in Table 3.3. For the female rats, there were no significant differences in haemoglobin concentrations ($p=0.87$), the haematocrit ($p=0.84$) and mean cell haemoglobin concentrations ($p=0.09$) across the different dietary groups.

Table 3. 3: Effects of 4-hydroxyisoleucine on the haemoglobin concentrations and haematocrit of the female rats after 10 weeks

	PDW + PGC	FDW + PGC	FDW + 4HGC	PDW + 4HGC	FDW + FENGC
Haemoglobin (g/dL)	13.94±1.54 [a]	14.28±1.67 [a]	14.63±2.58 [a]	15.09±2.76 [a]	14.19±2.48 [a]
Haematocrit (%)	41.63±4.53 [a]	42.75±4.83 [a]	43.88±7.68 [a]	45.38±8.30 [a]	42.75±7.50 [a]
Mean cell Haemoglobin (g/dL)	33.48±0.19 [a]	33.38±0.19 [a]	33.33±0.25 [a]	33.25±0.19 [a]	33.19±0.24 [a]

Data is presented as mean ± SD; n=8 females per dietary group. [a] Within row means with similar superscripts are not significantly different at $p>0.05$. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

The haemoglobin concentrations and haematocrit of the male rats are illustrated in Table 3.4. There were similarly no significant differences in haemoglobin concentrations ($p=0.99$), the haematocrit ($p=0.99$) and mean cell haemoglobin concentrations ($p=0.77$) of the male rats.

Table 3. 4: Effects of 4-hydroxyisoleucine on the haemoglobin concentrations and haematocrit of the male rats after 10 weeks

	PDW + PGC	FDW + PGC	FDW + 4HGC	PDW + 4HGC	FDW + FENGC
Haemoglobin (g/dL)	14.78±3.21 [a]	14.26±2.38 [a]	14.25±2.02 [a]	14.31±2.32 [a]	14.64±2.56 [a]
Haematocrit (%)	44.38±9.69 [a]	42.75±7.25 [a]	42.88±6.18 [a]	43.00±7.03 [a]	43.88±7.68 [a]
Mean cell Haemoglobin (g/dL)	33.30±0.24 [a]	33.37±0.26 [a]	33.24±0.11 [a]	33.29±0.27 [a]	33.36±0.21 [a]

Data is presented as mean ± SD; n=8 males per dietary group. [a] Within row means with similar superscripts are not significantly different at $p>0.05$. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

3.3 Visceral organs

The visceral organ absolute masses (g) and relative masses (% BW) of the visceral organs as well as the lengths (mm) of small and large intestines of the female rats are illustrated in Table 3.5. There were no significant differences in absolute masses (g) of the heart (p=0.94), pancreas (p=0.28), small intestine (p=0.28), large intestine (p=0.05), stomach (p=0.08), kidneys (p=0.57) and visceral fat (p=0.29) across the different dietary groups for female rats. In the female rats, there were no significant differences in absolute caecal mass across the groups except for those that were administered fructose and 4-OH-Ile (FDW+4HGC) which had significantly smaller caecal absolute masses (g) (p=0.0009) compared to female rats administered the normal diet (PDW+PGC). There was no significant difference in the liver masses of the female rats however those administered fructose and Fenofibrate (FDW+FENGC) had the largest absolute liver masses. There were no significant differences in the relative masses (% BW) of the heart (p=0.71), pancreas (p=0.56), caecum (p=0.15), small intestine (p=0.36), large intestine (p=0.05), stomach (p=0.09), kidneys (p=0.47) and visceral fat (p=0.12) of the females. There were also no significant differences in the relative liver masses (%) of the female rats except those administered fructose and Fenofibrate (FDW+FENGC) which had the largest relative liver masses. There were no significant differences in the lengths (mm) of the small intestines (p=0.99) and the large intestines (p=0.31) of the female rats.

Table 3. 5: Effects of 4-hydroxyisoleucine on the visceral organs morphometry (absolute and relative to body weight) of the female rats across different dietary treatment groups after 10 weeks

Organ	PDW + PGC	FDW + PGC	FDW + 4HGC	PDW + 4HGC	FDW + FENGC
Heart (g)	1.04±0.07 [a]	1.07±0.08 [a]	1.07±0.12 [a]	1.05±0.04 [a]	1.08±0.14 [a]
Heart (% BW)	0.40±0.02 [a]	0.42±0.04 [a]	0.42±0.05 [a]	0.40±0.03 [a]	0.41±0.03 [a]
Pancreas (g)	107±0.15[a]	1.01±1.17 [a]	1.00±0.19 [a]	0.97±0.15 [a]	1.13±0.11[a]
Pancreas (% BW)	0.41±0.06 [a]	0.40±0.08 [a]	0.40±0.09 [a]	0.37±0.05 [a]	0.43±0.07 [a]
Liver (g)	7.08±0.42 [a]	7.98±0.90 [a]	7.70±0.72 [a]	7.29±0.55 [a]	9.29±0.91 [b]
Liver (%)	2.72±0.14 [a]	3.11±0.35 [a]	3.04±0.36 [a]	2.82±0.29 [a]	3.56±0.50 [b]
Small intestine (g)	6.40±0.50 [a]	6.56±0.48 [a]	6.87±0.40 [a]	6.66±.47 [a]	6.90±0.69 [a]
Small intestine (% BW)	2.47±0.22 [a]	2.57±0.32 [a]	2.71±0.22 [a]	2.56±0.09 [a]	2.63±0.29 [a]
Small intestine (mm)	1208.00 ± 23.75 [a]	1211.00 ± 52.56 [a]	1203.00 ± 10.35 [a]	1215.00 ± 41.50 [a]	1210.00 ± 80.89 [a]
Large intestine (g)	1.41±0.10 [a]	1.26±0.13 [a]	1.33±0.13 [a]	1.42±0.08 [a]	1.30±0.13 [a]
Large intestine (% BW)	0.54±0.05 [a]	0.49±0.05 [a]	0.52±0.05 [a]	0.55±0.03 [a]	0.50±0.04 [a]
Large intestine (mm)	211.30±7.91 [a]	200.60±14.00 [a]	206.30±15.06 [a]	210.00±5.98 [a]	208.80±6.94 [a]
Caecum (g)	1.05±0.14 [a]	0.91±0.07[ab]	0.81±0.11 [b]	0.99±0.12 [ac]	0.88±0.09 [bc]

Caecum (% BW)	0.40±0.05[a]	0.36±0.05[a]	0.32±0.05[a]	0.38±0.06[a]	0.33±0.03[a]
Stomach (g)	1.41±0.12 [a]	1.52±0.24 [a]	1.42±0.12 [a]	1.31±0.07 [a]	1.48±0.15[a]
Stomach (% BW)	0.54±0.04 [a]	0.59±0.11 [a]	0.56±0.07 [a]	0.50±0.02 [a]	0.56±0.04 [a]
Kidneys (g)	1.62±0.09 [a]	1.64±0.16 [a]	1.67±0.13 [a]	1.66±0.11 [a]	1.73±0.20 [a]
Kidneys (% BW)	0.62±0.03 [a]	0.64±0.06 [a]	0.65±0.03 [a]	0.64±0.05 [a]	0.66±0.03 [a]
Visceral fat (g)	10.85±3.38 [a]	12.97±4.05 [a]	12.83±3.67 [a]	9.94±1.52 [a]	13.14±4.76 [a]
Visceral fat (% BW)	4.14±1.16 [a]	4.97±1.20 [a]	4.97±1.10 [a]	3.82±0.51 [a]	4.87±1.27 [a]

Data is presented as mean ± SD; n=8 females per dietary group. [abc] Within row means with different superscripts are significantly different at $p<0.05$. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

The visceral organ absolute masses (g) and relative masses (% BW) of the visceral organs as well as the lengths (mm) of small and large intestines of the male rats are illustrated in Table 3.6. There were no significant differences in absolute masses (g) of the heart (p=0.66), pancreas (p=0.94), small intestine (p=0.66), large intestine (p-0.05), caecum (p=0.05), stomach (p=0.05), testes (p=0.09), epididymal fat (p=0.89) and visceral fat (p=0.73) of the male rats. There were also no significant differences in absolute liver masses of the male rats except those administered fructose and Fenofibrate (FDW + FENGC) which had the largest liver masses (p=0.0001) compared to all other rats. There were no significant differences in absolute kidney masses however male rats administered fructose and 4-OH-Ile (FDW + 4HGC) had a significantly lower absolute kidney mass (g) (p=0.0045) compared to the male rats administered the normal diet (PDW + PGC).There were no significant differences in relative masses (% BW) of the pancreas (p=0.37), large intestine (p=0.96), kidneys (p=0.25), testes (p=0.83), epididymal fat (p=0.89) and visceral fat (p=0.29). There were also no significant differences in the relative heart masses of the male rats however male rats administered fructose and 4-OH-Ile (FDW + 4HGC) had a greater relative heart mass (% BW) (p=0.03) compared to the male rats administered plain drinking water and 4-OH-Ile (PDW + 4HGC). There was no significant difference in relative liver masses of the male rats except those administered fructose and Fenofibrate (FDW+FENGC) which had the largest relative liver mass (p=0.0001) compared to all the other rats. There were no significant differences in the relative small intestine masses however, male rats administered fructose and 4-OH-Ile (FDW + 4HGC) had a greater relative small intestine mass (% BW) (p=0.04) compared to male rats administered the normal diet (PDW + PGC). No significant differences were found in relative caecal masses however the male rats administered fructose and 4-OH-Ile (FDW+4HGC) had significantly smaller relative caecal masses (%) (p=0.009) compared to the male rats on the normal diet (PDW+PGC). There were no significant differences in relative stomach masses except those administered fructose and 4-OH-Ile (FDW + 4HGC) which had greater relative stomach masses (p=0.0001) compared to male rats administered plain drinking water and 4-OH-Ile (PDW + 4HGC) and male rats administered the normal diet (PDW + PGC) (p=0.0001). There were no significant differences in the lengths (mm) of the small intestines (p=0.11) and the large intestines (p=0.52) of the male rats.

Table 3. 6: Effects of 4-hydroxyisoleucine on the visceral organs morphometry (absolute and relative to body weight) of the male rats across different dietary treatment groups after 10 weeks

Organ	PDW + PGC	FDW + PGC	FDW + 4HGC	PDW + 4HGC	FDW + FENGC
Heart (g)	1.57±0.17 [a]	1.49±0.15 [a]	1.49±0.25 [a]	1.44±0.09 [a]	1.49±0.15 [a]
Heart (% BW)	0.36±0.03 [ab]	0.37±0.03 [ab]	0.39±0.04 [a]	0.35±0.02 [b]	0.37±0.03 [ab]
Pancreas (g)	1.44±0.23 [a]	1.45±0.28 [a]	1.45±0.39 [a]	1.37±0.17 [a]	1.48±0.22 [a]
Pancreas (% BW)	0.33±0.05 [a]	0.36±0.08 [a]	0.38±0.05 [a]	0.33±0.05 [a]	0.37±0.06 [a]
Liver (g)	12.52±1.00 [a]	12.04±1.02 [a]	11.32±1.96 [a]	11.22±0.50 [a]	15.08±1.30 [b]
Liver (%)	2.87±0.29 [a]	2.95±0.10 [a]	2.99±0.27 [a]	2.71±0.22 [a]	3.73±0.38 [b]
Small intestine (g)	9.16±0.78 [a]	9.40±0.69 [a]	9.13±0.77 [a]	8.99±0.75 [a]	9.47±0.59 [a]
Small intestine (% BW)	2.09±0.09 [a]	2.32±0.24 [a]	2.44±0.32 [a]	2.17±0.21 [a]	2.35±0.28 [a]
Small intestine (mm)	1373.00 ± 93.69 [a]	1370.00 ± 45.90 [a]	1306.00 ± 65.27 [a]	1372.00 ± 21.37 [a]	1455.00 ± 200.60 [a]
Large intestine (g)	1.90±0.33 [a]	1.77±0.23 [a]	1.57±0.11 [a]	1.87±0.17 [a]	1.77±0.21 [a]
Large intestine (% BW)	0.43±0.07 [a]	0.44±0.07 [a]	0.42±0.07 [a]	0.45±0.04 [a]	0.44±0.08 [a]
Large intestine (mm)	235.00±13.09 [a]	225.00±19.27 [a]	220.60±13.21 [a]	225.00±21.21 [a]	228.80±15.29 [a]

Caecum (g)	1.48±0.26[a]	1.19±0.18 [a]	1.18±0.28 [a]	1.45±0.24 [a]	1.33±0.27 [a]
Caecum (% BW)	0.34±0.06 [a]	0.29±0.04 [ab]	0.31±0.03 [b]	0.35±0.06 [ab]	0.33±0.05 [ab]
Stomach (g)	1.83±0.17[a]	1.97±0.11 [a]	1.89±0.26[a]	1.72±0.15[a]	1.97±0.19 [a]
Stomach (% BW)	0.42±0.03[a]	0.48±0.04[b]	0.50±0.04[b]	0.42±0.03[a]	0.49±0.05[b]
Kidneys (g)	2.67±0.27[a]	2.49±0.23[ab]	2.27±0.26[b]	2.55±0.09[ab]	2.66±0.18[a]
Kidneys (% BW)	0.61±0.06 [a]	0.61±0.04 [a]	0.60±0.06 [a]	0.62±0.05 [a]	0.66±0.05 [a]
Testes (g)	6.05±0.60 [a]	5.65±0.94 [a]	5.18±0.52 [a]	5.54±0.54 [a]	5.77±0.27 [a]
Testes (% BW)	1.38±0.06 [a]	1.38±0.20 [a]	1.40±0.25 [a]	1.33±0.11 [a]	1.43±0.14 [a]
Epididymal fat (g)	3.32±0.83 [a]	2.97±0.53 [a]	3.17±1.75 [a]	3.13±0.95 [a]	3.51±1.14 [a]
Epididymal fat (% BW)	0.75±0.15 [a]	0.73±0.11 [a]	0.79±0.29 [a]	0.75±0.19 [a]	0.85±0.22 [a]
Visceral fat (g)	11.15±3.10 [a]	11.90±3.02 [a]	11.63±6.16 [a]	9.52±2.89 [a]	12.06±4.04 [a]
Visceral fat (% BW)	2.50±0.47 [a]	2.89±0.56 [a]	2.90±1.05 [a]	2.27±0.58 [a]	2.92±0.80 [a]

Data is presented as mean ± SD; n=8 males per dietary group. [abc] Within row means with different superscripts are significantly different at $p<0.05$. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

3.4 Fasting blood glucose concentrations, plasma insulin concentrations, plasma adiponectin concentrations and insulin resistance

The fasting blood glucose concentrations, plasma insulin concentrations, plasma adiponectin concentrations and HOMA-IR values of the female rats are illustrated in Table 3.7. There was no significant difference in the fasting blood glucose concentrations ($p=0.83$) across the dietary groups. There was also no significant difference in the insulin concentration across the groups except for the female rats administered fructose and Fenofibrate (FDW+FENGC) which had a significantly higher plasma insulin concentration ($p=0.01$) compared to the female rats on the normal diet (PDW+PGC). There were no significant differences in the plasma adiponectin concentrations ($p=0.43$) of the female rats. The HOMA-IR ($p=0.18$) of the female rats in the different treatment groups were not significantly different.

Table 3. 7: Effects of 4-hydroxyisoleucine on fasting blood glucose concentrations, plasma insulin concentrations, plasma adiponectin concentrations and HOMA-IR indexes of the female rats after the 10 week treatment intervention

	PDW+PGC	FDW+PGC	FDW+4HGC	PDW+4HGC	FDW+FENGC
Glucose (mmol/L)	3.75±0.59[a]	3.60±0.65 [a]	3.63±0.57 [a]	3.84±0.46 [a]	3.85±0.38 [a]
Insulin (ng/mL)	1.59±0.63 [a]	1.73±0.41 [ab]	1.98±0.31 [ab]	2.10±0.27 [ab]	2.38±0.42 [b]
Adiponectin (pg/mL)	27.37±22.52 [a]	15.57±9.272 [a]	26.26±21.62 [a]	25.91±14.48 [a]	35.64±16.13 [a]
HOMA-IR	4.75±2.97[a]	5.55±1.82[a]	6.42±1.57[a]	7.12±0.92[a]	7.06±3.12[a]

Data is presented as mean ± SD; n=8 females per dietary group. [ab] Within row means with different superscripts are significantly different at $p<0.05$. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

The fasting blood glucose concentrations, plasma insulin concentrations, plasma adiponectin concentrations and HOMA-IR values of the male rats are illustrated in Table 3.8. There was no significant difference in the fasting blood glucose concentrations ($p=0.16$) as well as the plasma insulin concentrations ($p=0.61$) of the male rats across the dietary treatment groups. There were no significant differences in the plasma adiponectin concentrations ($p=0.64$) of the male rats. The HOMA-IR ($p=0.57$) of all the male rats was not significantly different.

Table 3. 8: Effects of 4-hydroxyisoleucine on fasting blood glucose concentrations, plasma insulin concentrations, plasma adiponectin concentrations and HOMA-IR indexes of the male rats after the 10 week treatment intervention

	PDW+PGC	FDW+PGC	FDW+4HGC	PDW+4HGC	FDW+FENGC
Glucose (mmol/L)	3.68±0.27 [a]	3.76±0.75 [a]	3.46±0.46 [a]	3.73±0.51 [a]	4.26±0.49 [a]
Insulin (ng/mL)	1.53±0.55 [a]	1.76±0.57 [a]	1.93±0.32 [a]	1.93±0.64 [a]	1.79±0.65 [a]
Adiponectin (pg/mL)	13.34±12.90 [a]	17.08±13.75 [a]	17.58±18.66 [a]	23.94±9.99 [a]	24.99±17.28 [a]
HOMA-IR	5.04± 1.93[a]	5.90± 2.33[a]	5.91± 1.17[a]	6.47± 2.47[a]	6.72± 2.47[a]

Data is presented as mean ± SD; n=8 males per dietary group. [a] Within row means with similar superscripts are not significantly different at $p>0.05$. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

3.5 Blood lipids (total cholesterol and triglycerides) of the female and male rats after the 10-week treatment intervention

The total cholesterol concentrations and triglyceride concentrations of the female rats are shown in Figures 3.7 and 3.8 respectively. There was no significant difference in cholesterol concentrations ($p=0.43$) across the dietary groups. There was no significant difference in plasma triglyceride concentrations of the female rats except those administered Fenofibrate and fructose (FDW+FENGC) which had significantly lower ($p=0.0038$) triglyceride concentrations compared to rats administered plain drinking water and 4-OH-Ile (PDW+4HGC) and also compared to rats on the normal (PDW+PGC) diet.

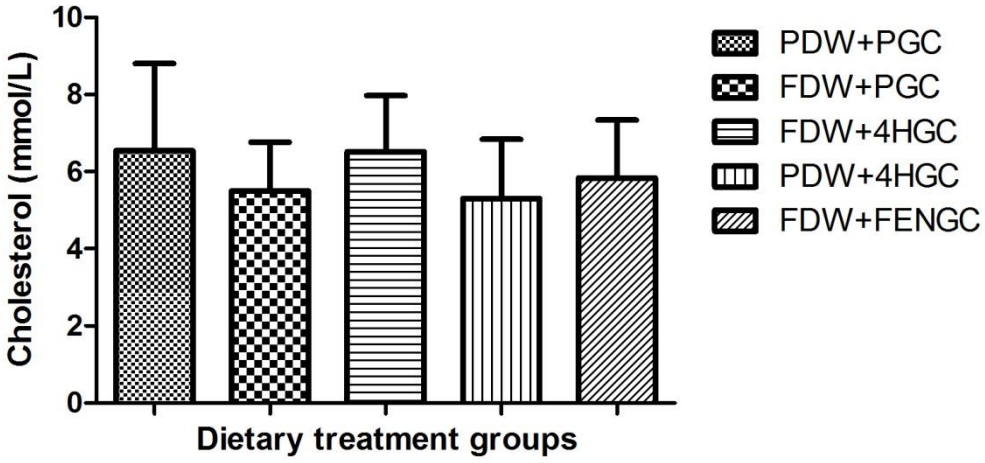


Figure 3. 7: The effects of 4-hydroxyisoleucine on plasma total cholesterol concentrations of the female rats across the different dietary treatment groups.

Data is presented as mean ± SD; n=8 females per dietary group. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

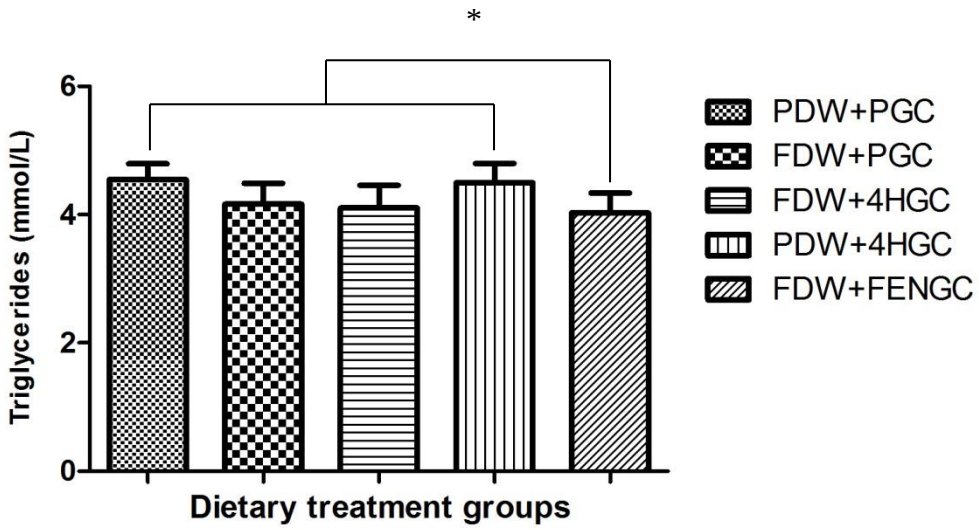


Figure 3. 8: The effects of 4-hydroxyisoleucine on plasma triglyceride concentrations of the female rats across the different dietary treatment groups.

Data is presented as mean ± SD; n=8 females per dietary group. * $p < 0.05$. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

The total cholesterol concentrations and triglyceride concentrations of the male rats are shown in Figures 3.9 and 3.10 respectively. There was no significant differences in total cholesterol concentrations ($p=0.57$) as well as triglyceride concentrations ($p=0.055$) of the male rats across all the experimental groups.

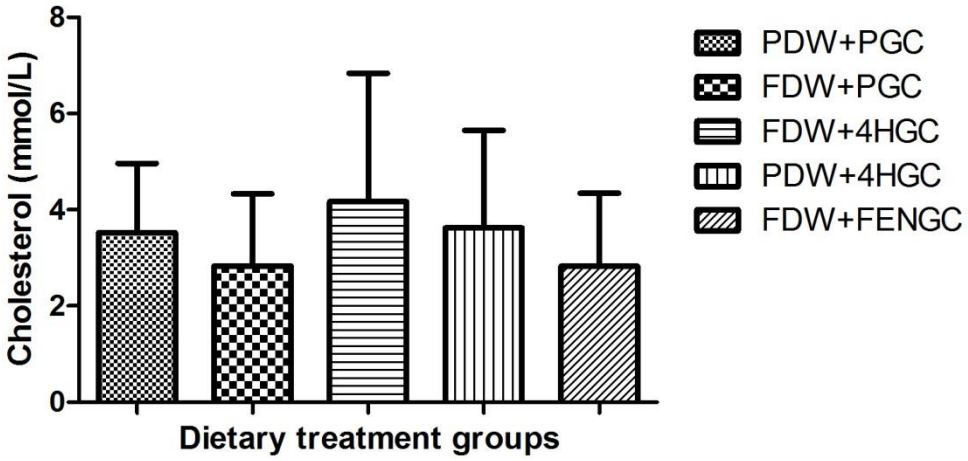


Figure 3. 9: The effects of 4-hydroxyisoleucine on plasma total cholesterol concentrations of the male rats across the different dietary treatment groups.

Data is presented as mean ± SD; n=8 males per dietary group. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

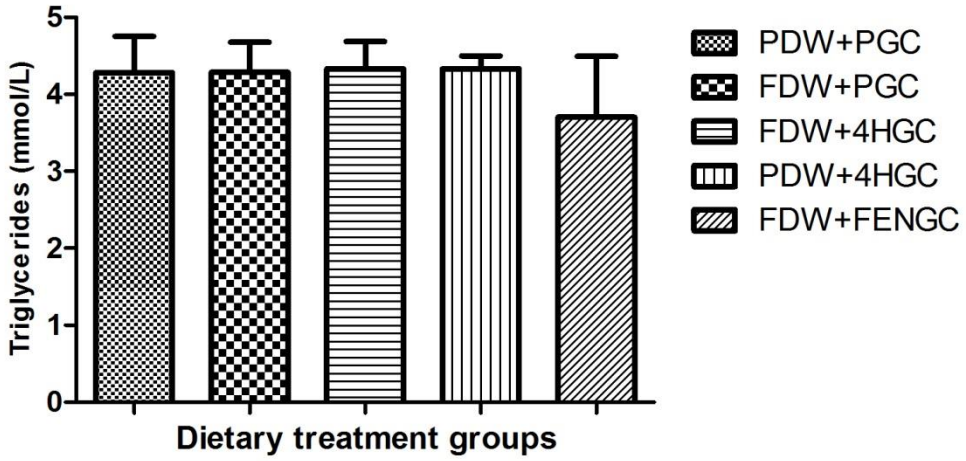


Figure 3. 10: The effects of 4-hydroxyisoleucine on plasma triglyceride concentrations of the male rats across the different dietary treatment groups.

Data is presented as mean ± SD; n=8 males per dietary group. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

3.6 The development of non-alcoholic fatty liver disease of the female and male rats after the 10-week treatment intervention

The NAFLD scores in the female rats for the different dietary groups are illustrated in Table 3.9. The representative photomicrographs for H and E or Masson's trichrome liver histology for the dietary groups of the female rats are shown in Figures 3.11 and 3.12 respectively. One representative picture was randomly selected from each group. There were no significant differences in the macrovesicular steatosis scores of females however, female rats administered fructose (FDW+PGC) had significantly more macrovesicular steatosis ($p<0.05$) as compared to all other female rats. There were no significant differences in microvesicular steatosis scores of the females except for those administered fructose (FDW+PGC) which had significantly greater microvesicular steatosis ($p=0.01$) compared to female rats administered plain drinking water and 4-OH-Ile (PDW + 4HGC) and those administered fructose and Fenofibrate (FDW+FENGC). There was no significant difference on the hypertrophy scores ($p>0.05$) of the hepatocytes across all the dietary groups. In addition, there was no significant difference in inflammation ($p=0.08$) and fibrosis ($p=0.06$) scores of the liver samples in all the female rats across the different dietary groups.

Table 3. 9: Effects of 4-hydroxyisoleucine on the development of NAFLD in female rats after the 10-week treatment intervention

	PDW+PGC	FDW+PGC	FDW+4HGC	PDW+4HGC	FDW+FENGC
Macrovesicular steatosis	1.00(0;0)[a]	0.00(0;1)[b]	0.00(0;0)[a]	0.00(0;0)[a]	0.00(0;0)[a]
Microvesicular steatosis	0.00(0;1)[ab]	1.50(1;3)[a]	0.50(0;1)[ab]	0.00(0;0)[bc]	0.00(0;0)[bc]
Hypertrophy	0.00(0;0) [a]	1.00(1;1) [a]	0.00(0;0) [a]	0.50(0;1) [a]	1.00(0;1) [a]
Inflammation	0.00(0;0) [a]	1.00(1;1) [a]	0.50(0;1) [a]	0.50±(0;1) [a]	0.00(0;1) [a]
Fibrosis (%)	9.69±2.55 [a]	6.58±1.49[a]	6.68±2.78 [a]	4.53±0.94 [a]	5.31±2.32 [a]

Non-parametric data is presented as median(IQR). Parametric data is presented as mean ± SD; n=4 females per dietary group. [abc] Within row means with different superscripts are significantly different at $p<0.05$. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid drinking water and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

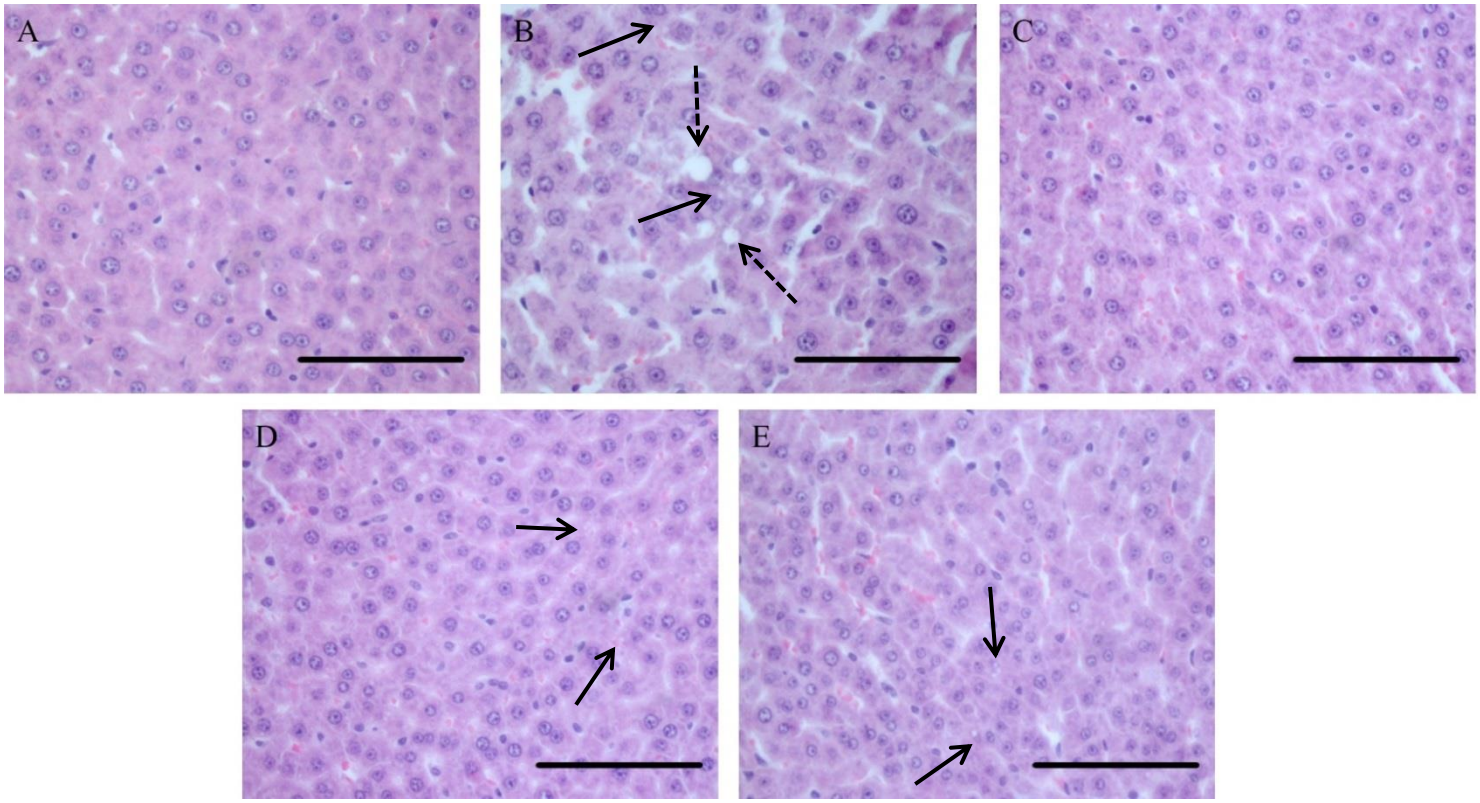


Figure 3. 11: Representative histological sections of female liver samples stained with haematoxylin and eosin at 40 times magnification.

Scale bar = 20µm, applies to all the figures. Solid black arrow indicates microvesicular steatosis and broken-line black arrow indicates macrovesicular steatosis. A-PDW + PGC= plain drinking water and plain gelatine cube; B-FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; C-FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); D-PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); E-FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

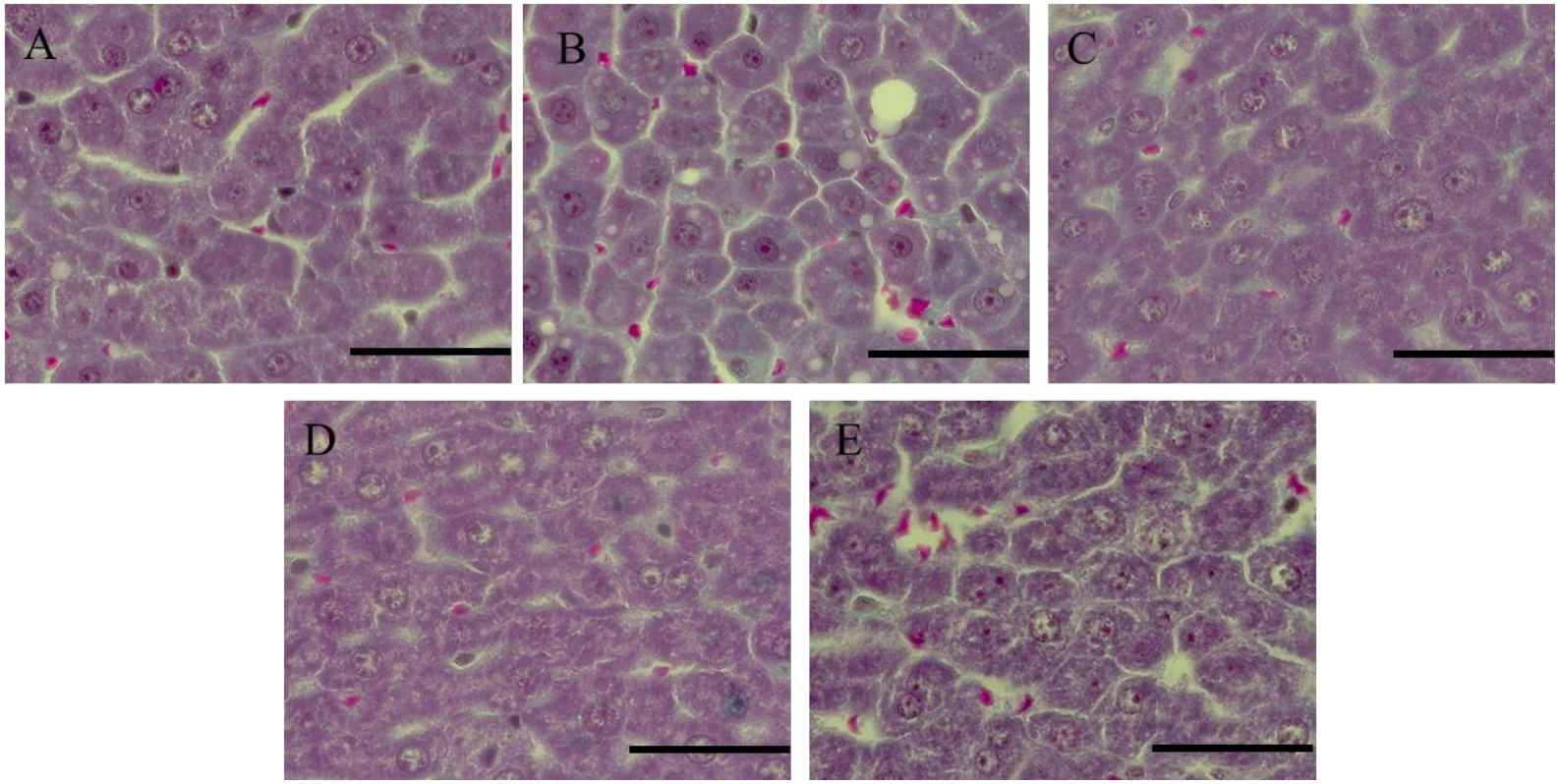


Figure 3. 12: Representative histological sections of female liver samples stained with Masson's trichrome at 100 times magnification.

Scale bar in FDW+FENGC = 20µm, applies to all the figures. A-PDW + PGC= plain drinking water and plain gelatine cube; B-FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; C-FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); D-PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); E-FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

The NAFLD scores in the male rats for the different dietary groups are illustrated in Table 3.10. The representative photomicrographs for H and E or Masson's trichrome liver histology for the dietary groups of the male rats are shown in Figure 3.13 and 3.14 respectively. One picture was randomly selected from each group. There was no significant difference in macrovesicular ($p=0.44$) and microvesicular ($p=0.26$) hepatic steatosis scores of the male rats. In addition, there was no significant difference in hypertrophy ($p=0.19$), inflammation ($p=0.44$) and fibrosis ($p=0.90$) in the liver samples of male rats.

Table 3. 10: Effects of 4-hydroxyisoleucine on the development of NAFLD in male rats after the 10-week treatment intervention

	PDW+PGC	FDW+PGC	FDW+4HGC	PDW+4HGC	FDW+FENGC
Macrovesicular steatosis	0.00(0;0) [a]	0.00(0;0) [a]	0.00(0;0) [a]	0.00(0.1) [a]	0.00(0;0) [a]
Microvesicular steatosis	0.00(0;1) [a]	2.00(0;2) [a]	0.50±(0;1) [a]	0.00(0;0) [a]	0.50(0;1) [a]
Hypertrophy	0.00(0;0) [a]	1.00(0;1) [a]	0.00(0;1) [a]	0.00(0;0) [a]	0.50(0;1) [a]
Inflammation	0.00(0;1) [a]	1.00(1;1) [a]	0.50(0;1) [a]	0.50(0;1) [a]	0.50(0;1) [a]
Fibrosis (%)	5.56±1.038 [a]	4.69±1.05 [a]	5.76±0.93 [a]	5.46±1.44 [a]	6.34±3.35 [a]

Non-parametric data is presented as median(IQR). Parametric data is presented as mean ± SD; n=4 males per dietary group. [a] Within row means with similar superscripts are significantly not different at $p>0.05$. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

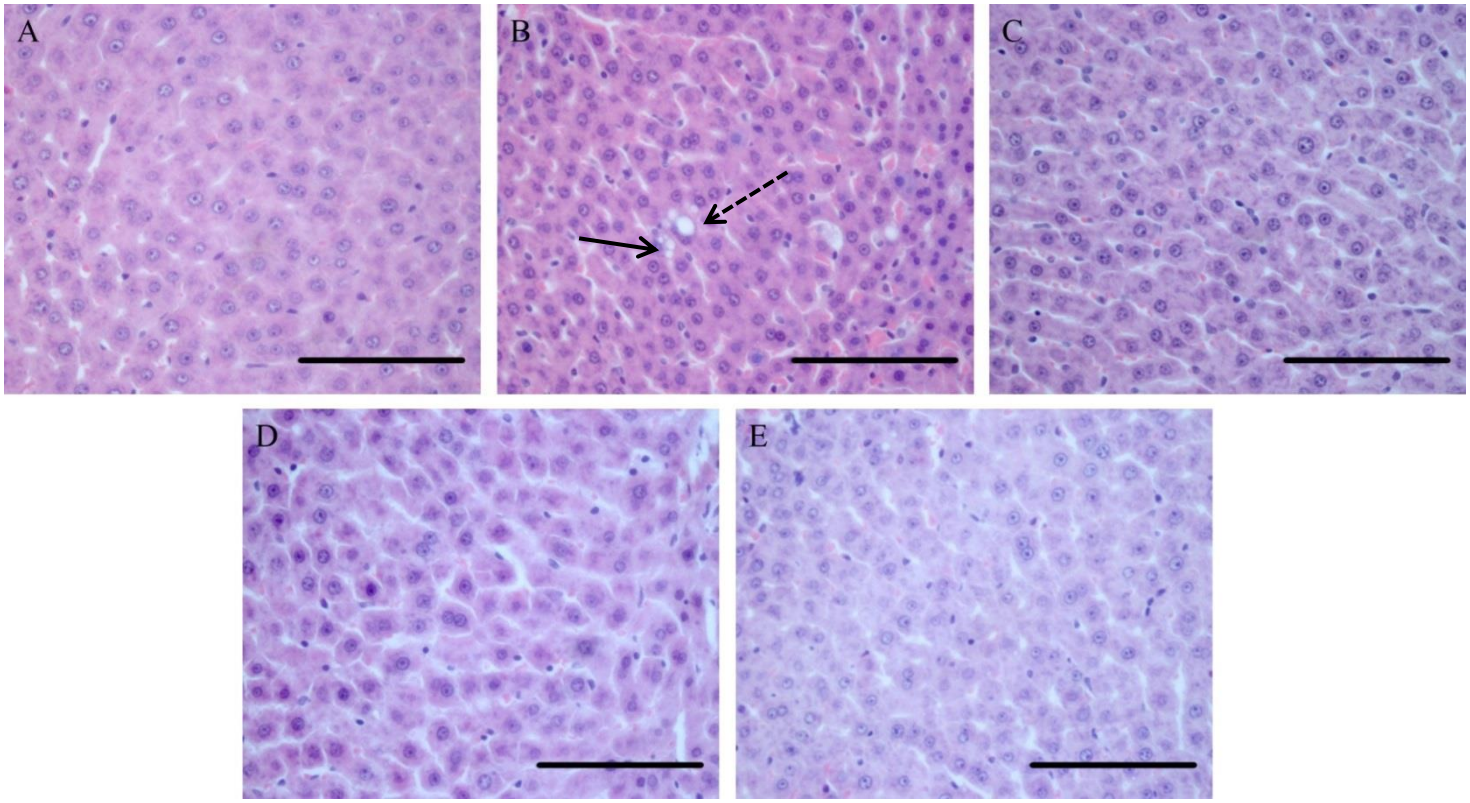


Figure 3. 13: Representative histological sections of male liver samples stained with haematoxylin and eosin at 40 times magnification.

Scale bar = 20µm, applies to all the figures. Solid black arrow indicates microvesicular steatosis and broken-line black arrow indicates macrovesicular steatosis. A-PDW + PGC= plain drinking water and plain gelatine cube; B-FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; C-FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); D-PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); E-FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

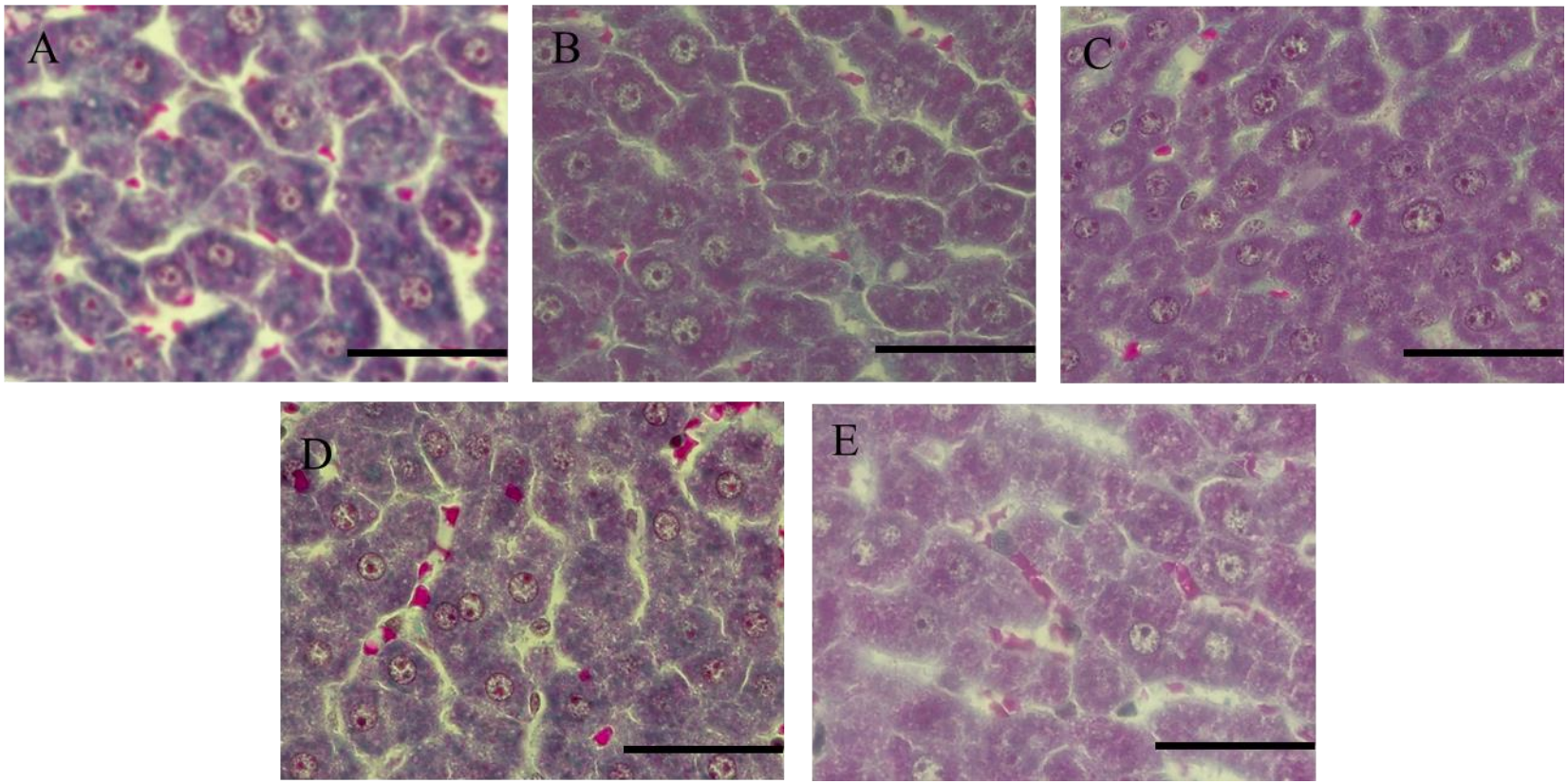


Figure 3. 14: Representative histological sections of male liver samples stained with Masson's trichrome at 100 times magnification.

Scale bar = 20µm, applies to all the figures. A-PDW + PGC= plain drinking water and plain gelatine cube; B-FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; C-FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); D-PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); E-FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

3.7 Kidney morphometry of the female and male rats after the 10-week treatment intervention

The renal corpuscle area (A_R), glomerulus area (A_G) and area of the Bowman's capsule space (A_B) of female rats are illustrated in Table 3.11. The representative photomicrographs of each experimental group are illustrated in Figures 3.15 and 3.16 for H and E and MT staining respectively. One picture was randomly selected from each group. There was no significant difference in the A_R of female rats except for those administered fructose (FDW+PGC) and fructose and Fenofibrate (FDW+FENGC) which had a significantly smaller A_R ($p=0.01$) compared to female rats in the normal diet (PDW+PGC) group. There was no significant difference ($p=0.05$) in the area of the A_G of female rats. The A_B of female rats was also not significant. However, female rats administered fructose and Fenofibrate (FDW+FENGC) had a significantly smaller ($p=0.0001$) A_B than the rats on the normal diet (PDW+PGC) or those administered fructose (FDW+PGC) ($p=0.001$). Across the groups in the female rats, fibrosis was not significantly different ($p=0.07$).

Table 3. 11: Effects of 4-hydroxyisoleucine on the kidney morphometry of the female rats after the 10-week treatment intervention

	PDW+PGC	FDW+PGC	FDW+4HGC	PDW+4HGC	FDW+FENGC
A_R (µm²)	10469±2481[a]	8471±2254[b]	8836±2881[ab]	9657±3622[ab]	8558±2407[b]
A_G (µm²)	6540±1809[a]	5368±2038[a]	5893±2108[a]	5686±2576[a]	6528±2723[a]
A_B (µm²)	3929±1534[a]	3103±1405[a]	2944±2088[ab]	3971±2310[a]	2029±1809[b]
Fibrosis (%)	3.642±1.500 [a]	3.827±2.384 [a]	6.276±1.517 [a]	2.562±0.8867 [a]	3.025±1.445 [a]

Data is presented as mean ± SD; n=4 females per dietary group. [ab] Within row means with different superscripts are significantly different at $p<0.05$. A_R = area of renal corpuscle, A_G = area of glomerulus, A_B = area of Bowman's capsule space. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid drinking water and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

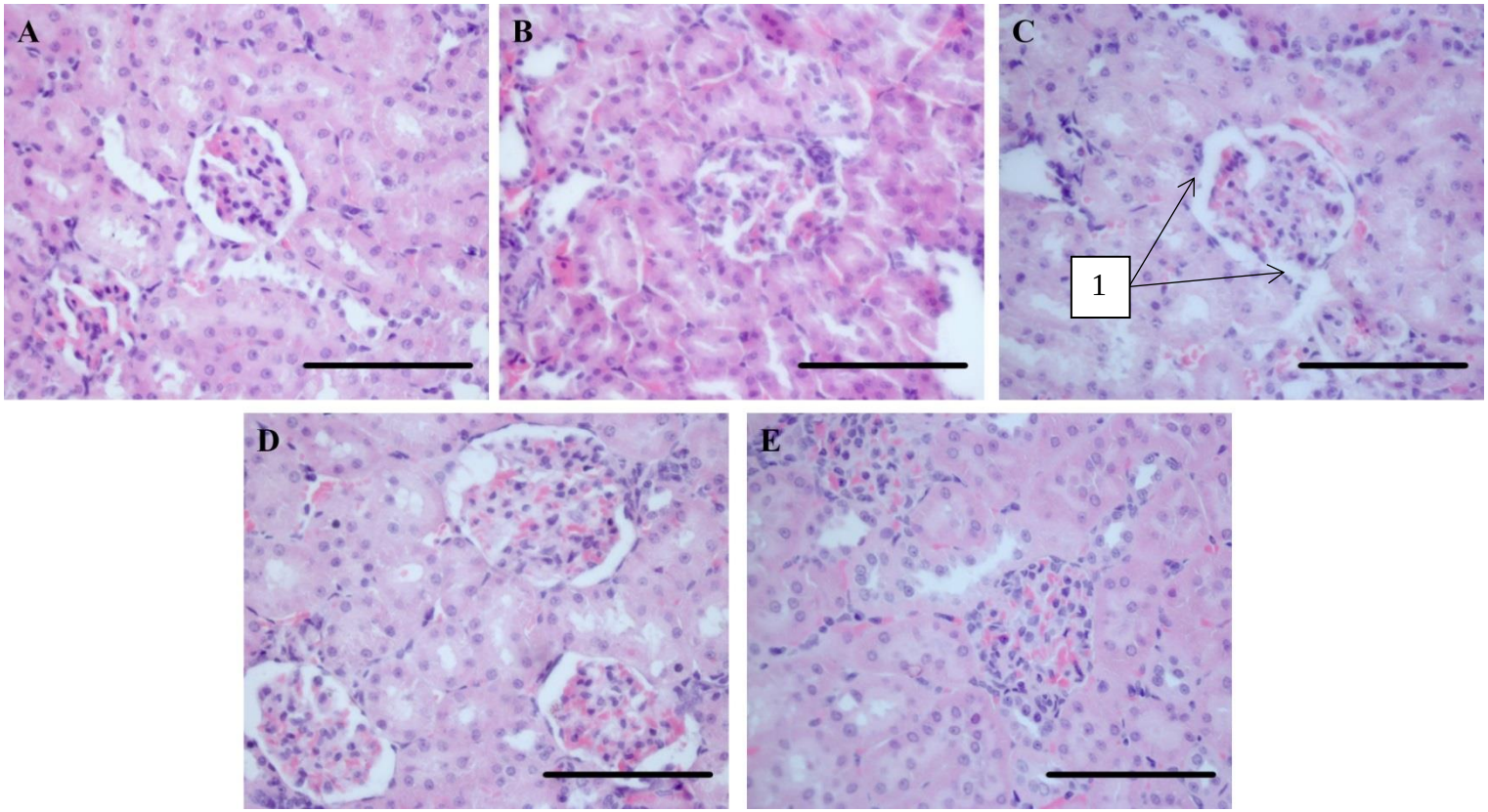


Figure 3. 15: Representative histological sections of female kidney samples stained with Haematoxylin and Eosin stain at 40 times magnification.

Scale = 20µm, applies to all the figures. 1= Renal corpuscle. A-PDW + PGC= plain drinking water and plain gelatine cube; B-FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; C-FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); D-PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); E-FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

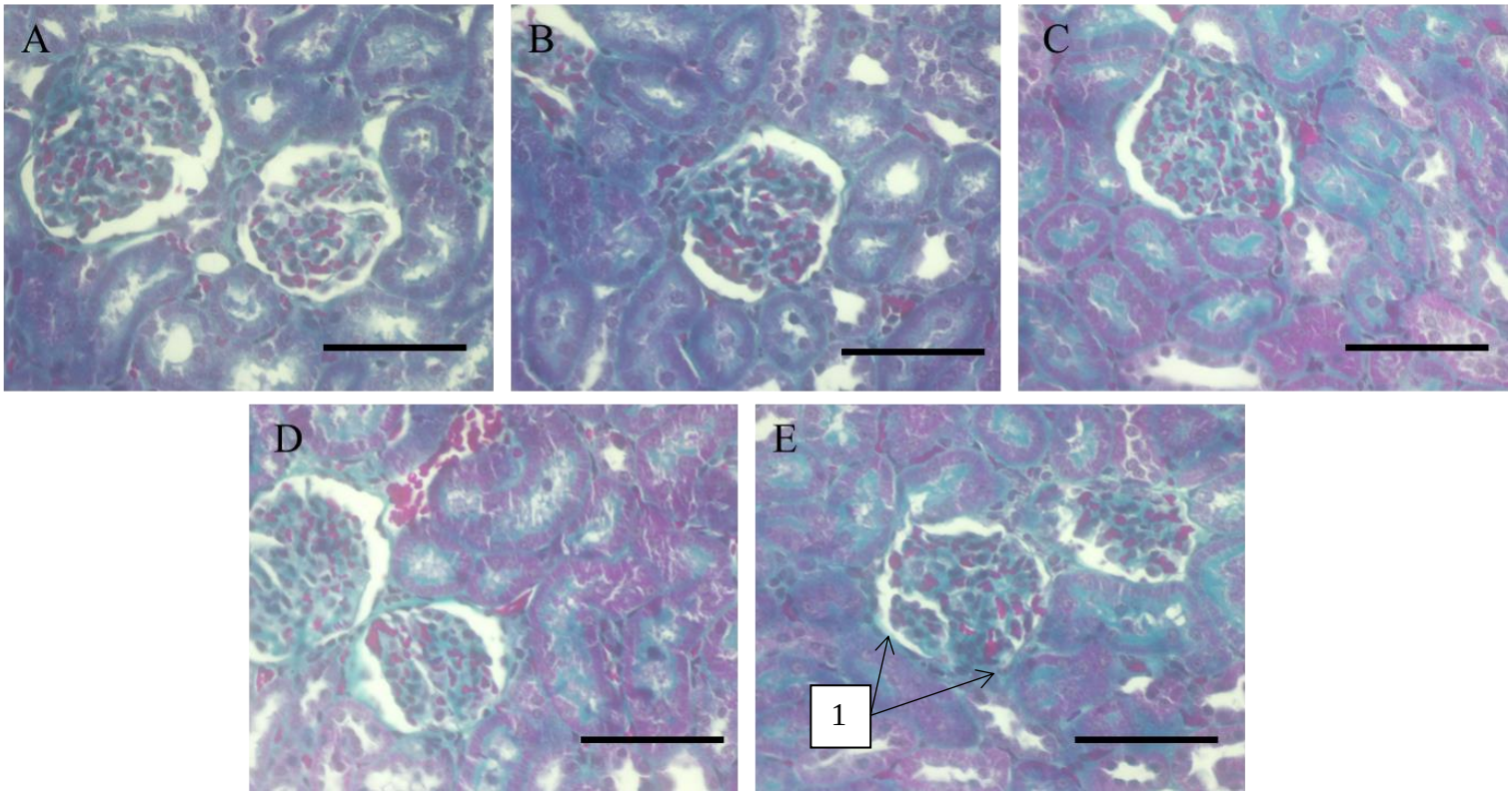


Figure 3. 16: Representative histological sections of female kidney samples stained with Masson's trichrome stain at 40 times magnification.

Scale = 20µm, applies to all the figures. 1= Renal corpuscle. A-PDW + PGC= plain drinking water and plain gelatine cube; B-FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; C-FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); D-PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); E-FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

The renal corpuscle area (A_R), glomerulus area (A_G) and Bowman's capsule space area (A_B) of male rats are illustrated in Table 3.12. The representative photomicrographs of each group are illustrated in Figures 3.17 and 3.18 for H and E and MT staining respectively. One picture was randomly selected from each group. There was no significant difference in the A_R ($p=0.0001$) of male rats except male rats administered the normal diet (PDW+PGC) which had the largest A_R. There were no significant differences in the A_G of male rats except the male rats administered fructose (FDW+PGC), those administered fructose and 4-OH-Ile (FDW + 4HGC) and those administered plain drinking water and 4-OH-Ile (PDW + 4HGC) which had significantly smaller ($p=0.0016$) A_G compared to male rats on the normal diet (PDW+PGC). There was no significant difference in the A_B of the males however male rats administered fructose and 4-OH-Ile (FDW + 4HGC) and those administered plain drinking water and 4-OH-Ile (PDW + 4HGC) had a significantly smaller A_B ($p=0.0001$) compared to male rats administered fructose and Fenofibrate (FDW + FENGC). Male rats on the normal diet (PDW + PGC) had the largest A_B compared to other groups. Across the groups in the male rats, fibrosis was not significantly different ($p=0.27$).

Table 3. 12: Effects of 4-hydroxyisoleucine on the kidney morphometry of the male rats after the 10-week treatment intervention

	PDW+PGC	FDW+PGC	FDW+4HGC	PDW+4HGC	FDW+FENGC
A_R (µm²)	14757±5552^{a}	10469±2815^{b}	9543±3338^{bc}	10007±3088^{bd}	11472±3943^{be}
A_G(µm²)	8902±3642^{a}	6847±2146^{b}	6416±2239^{bc}	7058±2389^{bd}	7135±3109^{ab}
A_B (µm²)	5855±2672^{a}	3622±1542^{b}	3126±2130^{bc}	2949±1701^{bd}	4337±2131^{be}
Fibrosis (%)	2.551±0.3772 ^{a}	2.551±0.3772 ^{a}	5.864±3.073 ^{a}	3.580±2.775 ^{a}	5.741±3.376 ^{a}

Data is presented as mean ± SD; n=4 males per dietary group. ^{abcde} Within row means with different superscripts are significantly different at $p<0.05$. A_R = area of renal corpuscle, A_G = area of glomerulus, A_B = area of Bowman's capsule space. PDW + PGC= plain drinking water and plain gelatine cube; FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); FDW + FENGC = 20% fructose solution as drinking fluid drinking water and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

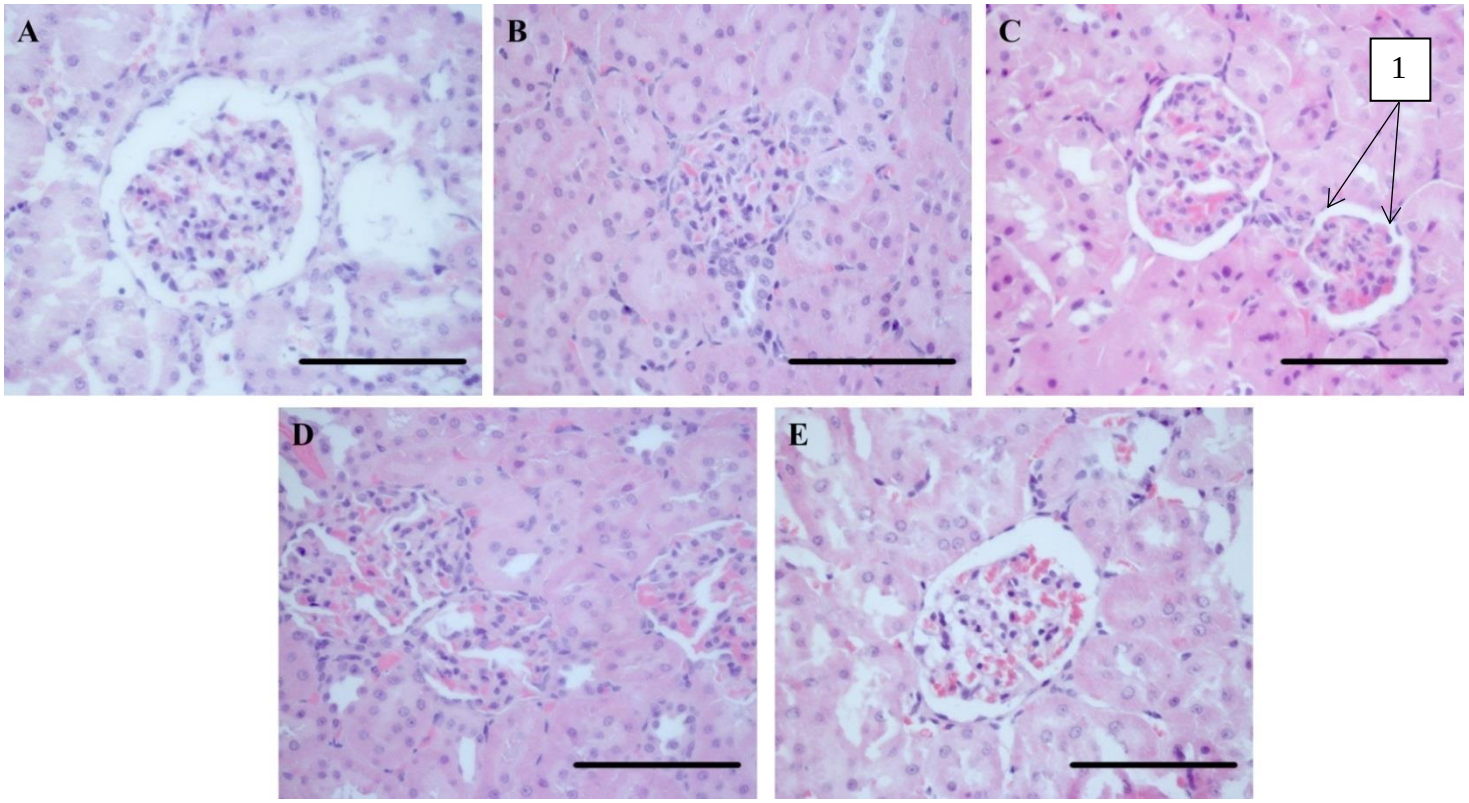


Figure 3. 17: Representative histological sections of male kidney samples stained with Haematoxylin and Eosin stain at 40 times magnification.

Scale = 20µm, applies to all the figures. 1= Renal corpuscle. A-PDW + PGC= plain drinking water and plain gelatine cube; B-FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; C-FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); D-PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); E-FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

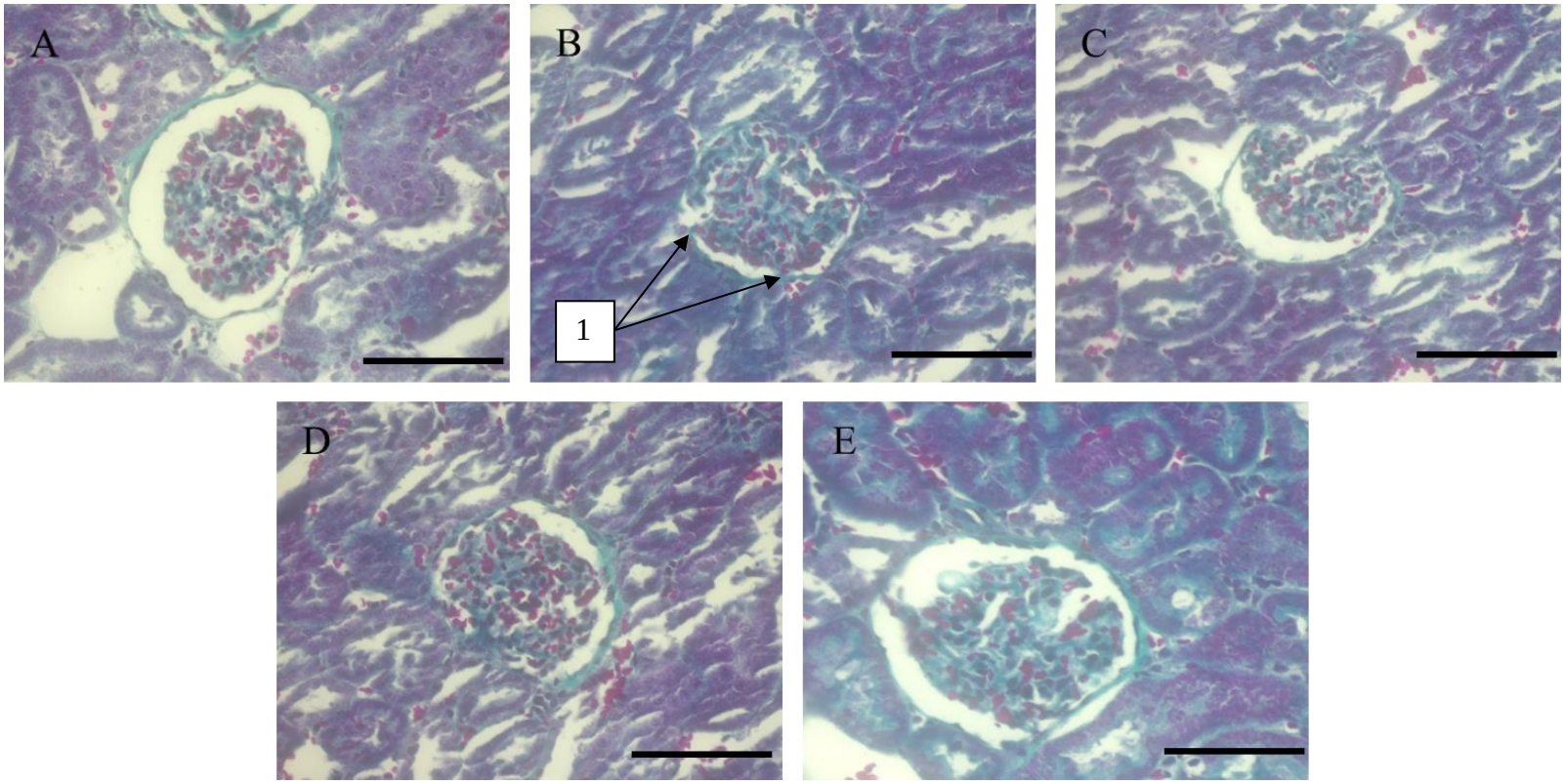


Figure 3. 18: Representative histological sections of male kidney samples stained with Masson's trichrome stain at 40 times magnification.

Scale = 20µm, applies to all the figures. 1= Renal corpuscle. A-PDW + PGC= plain drinking water and plain gelatine cube; B-FDW + PGC= 20% fructose solution as drinking fluid and plain gelatine cube; C-FDW + 4HGC = 20% fructose solution as drinking fluid and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); D-PDW + 4HGC = plain drinking water and a gelatine cube containing 4-hydroxyisoleucine (500 mg/kg body mass per day); E-FDW + FENGC = 20% fructose solution as drinking fluid and a gelatine cube containing Fenofibrate (100 mg/kg body mass per day).

CHAPTER 4: DISCUSSION

4.1 Growth performance

4.1.1 Body weight and weight gain

Body weight is used as a measure or an index of obesity as well as an index of growth performance (Pullen, 1976). In the current study, induction body weights of female rats were similar. The terminal body weights of female rats were also similar as reflected in weight gain percentage over the experimental period. The male rats also showed similar trends except for those administered fructose and 4-OH-Ile which had a lower terminal body weight compared to the control group.

There are differing reports on the effect of high-fructose diets on body weight of rats. For example, Bocarsly et al. (2010) showed that a high-fructose diet caused an increase in body weights of female and male *Sprague Dawley* rats fed over 8 weeks compared to rats on a normal diet. While, Ozcan-Sinir et al. (2016) showed that daily feeding of a high-fructose diet for 7 months had no effect on body weight in male *Sprague Dawley* rats. Several factors such as the age of rats, mode of administration and feeding duration affect the response of rats to a high-fructose diet. First, a high-fructose diet is more effective in causing metabolic syndrome in adult rats than in young rats (de Moura et al., 2009). This could account for the differences in outcomes between reports. Second, some studies have found that adding fructose to the feed rather than drinking water is more effective in causing metabolic syndrome as fructose increases appetite and causes rats to eat more food rather than drink fluids (de Moura et al., 2009). Third, the effects of a high-fructose diet on body weight in rats will most likely not be observed if the dietary period is short term (i.e. < 6 weeks) (Rizkalla, 2010). Despite feeding the rats for more than 6 weeks through drinking water as in this study, there were no differences on body weight. In the current study young rats were used, fructose was added to drinking water instead of the feed and duration of feeding was not short term. These factors could have accounted for the lack of differences in body weights and body weight gains of the rats.

Despite the differences in the experimental designs e.g. short dietary period (8 weeks) and low fructose concentration (10%) compared to this study, Zhang et al. (2009) similarly showed that there was no significant difference in the body weights of adult (8 weeks old) male Wistar rats placed only on a high-fructose diet and those on a high-fructose diet supplemented with 4-OH-Ile at 50 mg/kg per day (Zhang et al., 2009). Metwally et al. (2017)

also showed that the administration of Fenofibrate (FF) to rats on a high-caloric diet causes a decrease in body weight (Metwally et al., 2017) contrary to the findings of this study. However, it must be noted that body weight is affected by differences in size of visceral organs, variations in the GIT contents in individual animals and hydration status of each animal (MacCracken and Stebbings, 2012; Owens et al., 1995). Thus, body weight is not considered the best indicator for long term growth performance (MacCracken and Stebbings, 2012; Owens et al., 1995). To eliminate these confounding factors, measurements of bones were used as indices of growth.

4.1.2 Long bone growth

Long bone (e.g. tibia, femur) growth is a good indicator of growth performance as long bones grow in response to growth hormone (GH) in a dose-dependent manner (Rol De Lama et al., 2000) and do not change in mass and in length acutely (K G Ibrahim et al., 2019). In the current study neither high-fructose diet alone nor oral administration of 4-OH-Ile and FF to rats fed a high-fructose diet had an effect on measured parameters of the bones i.e. tibia and femur of the rats.

Contrary to this study, female *Sprague Dawley* rats administered a high fructose diet for 8 weeks showed decreased tibia and femur masses according to the report by Tsanzi et al. (2008) while Ibrahim et al. (2019) reported no effect of high-fructose feeding on lengths, masses and densities of the tibia and femora of rats which is similar to the outcome of the present study. Despite no adverse or beneficial effects being observed on bone parameters measured in this study, the long term effects of 4-OH-Ile and FF on long bone growth in rats fed a high-fructose diet should be further investigated as there might be latent effects which could manifest in old age.

In light of the differences in reports, it seems when used singly, growth performance and body weight gains are not sufficient indices of obesity in animal studies (Ozcan Sinir et al., 2016). It is thus imperative to investigate other markers that could be used to determine the degree of adiposity.

4.2 Markers of adiposity

Adipose tissue is divided into two major regional depots namely subcutaneous adipose tissue and visceral adipose tissue (Yilmaz, 2012). In some studies visceral fat is reported as the sum combination of retroperitoneal fat, mesenteric and epididymal fat (Chusyd et al., 2016; Chun et al., 2010). However, in the current study visceral fat mass was separated from epididymal fat mass because epididymal fat is linked to infertility whereas other visceral fat components (mesenteric fat and retroperitoneal fat) are linked to metabolic syndrome (Hung et al., 2014; Katib, 2015). In this study, the masses of visceral and epididymal fat were measured to determine adiposity in rats.

4.2.1 Visceral and epididymal fat

Visceral fat accumulation has been reported to be directly associated with metabolic diseases (Metwally et al., 2017). The absolute and relative visceral fat masses of the female rats were similar and that of the male rats were also similar.

Previous studies have shown that the risk of developing high fructose diet–induced–obesity is sexually dimorphic. In a study by Sandeva et al. (2015), adult Wistar rats fed a high-fructose diet in drinking water (20% fructose) for 8 weeks, only female rats had an increase in visceral fat mass (Sandeva et al., 2015). On the contrary, the present study did not observe a difference in visceral fat masses of female rats despite similarities in the experimental designs used in this study and that used by Sandeva et al. (2015) e.g. same fructose concentration (20%) and similar duration of administration. However, Sandeva et al. (2015) used adult rats compared to the young (growing) rats used in this study. It has been reported that young rats have a higher basal metabolic rate than older rats which can be related to differences in body size and the need to generate energy needed for growth in young rats (Kleiber & Cole, 1950). Thus, this results in decreased visceral fat mass accretion when fed high fructose diets (Else and Hulbert, 2017; Tillman et al., 2014).

Handa et al. (2005) showed that the administration of fenugreek seed extract with 4-OH-Ile as the major component (20%) to adult female mice on a high-caloric diet decreased visceral fat masses of the mice (Handa et al., 2005). Although Handa et al. (2005) showed that 20% 4-OH-Ile added to the feed decreases visceral fat mass in male mice, a more pure compound of 98.5% 4-OH-Ile in gelatine cubes used in this study did not decrease the visceral fat in both

male and female rats. The differences observed in the two studies may be attributed to the presence of other components in the seed extract e.g. soluble fibre and diosgenin used in the study by Handa et al. (2005) compared to the purified form used in the present study. Soluble fibre and diosgenin have been shown to attenuate obesity (Fuller & Stephens, 2015).

Fenofibrate has been shown to prevent the increase in adiposity or visceral fat in a variety of animal models by acting on peroxisome proliferator-activated receptor activator α (PPAR α) in liver and adipose tissue thus increasing fatty acid catabolism by hepatic mitochondria and adipose tissue resulting in decreased visceral fat (Park et al., 2007). In this study, FF did not decrease visceral fat mass in either sex, most likely due to a non-significant impact of fructose.

Although there were no grossly observed differences in epididymal fat, there is a need in future studies to investigate the impact of interventions on sperm and semen quality and fertilising capacity. Aside the presence of visceral fat in adiposity, visceral fat has also been shown to secrete pro-inflammatory adipokines which can cause metabolic diseases (e.g. insulin resistance and type 2 diabetes) (Metwally et al., 2017; Pereira et al., 2017).

4.3 Hormones and metabolic substrates

4.3.1 Adiponectin

Adiponectin is exclusively secreted by adipocytes and decreases in the presence of metabolic syndrome (Kamari et al., 2007). In this study, adiponectin concentrations of female and male rats in the different dietary groups were not significantly different. In the current study no differences were observed in visceral fat accumulation which could explain the lack of changes in adiponectin concentrations.

4.3.2 Fasting glucose concentrations

Hyperglycaemia or increased fasting blood glucose levels are indicative of the presence of type 2 diabetes mellitus (Rizza, 2010). In this study, fasting blood glucose concentrations of females across the groups were similar. However, male rats administered a high-fructose diet and 4-OH-Ile had lower fasting blood glucose concentrations compared to male rats

administered fructose and FF. This suggests that 4-OH-Ile has hypoglycaemic properties. The potential hypoglycaemic mechanism(s) of action of 4-OH-Ile need to be further explored in future studies.

In other studies, a high-fructose diet has been shown to increase blood glucose concentrations. For example a high-fructose diet (10%) fed to adult male Wistar rats for 8 weeks was found to increase blood glucose concentrations (Zhang et al., 2009). There are studies, current study included, which have found no effect on blood glucose after fructose feeding: Ibrahim et al. (2019) reported that a high-fructose (20% fructose) diet fed to young rat pups for 51 days had no effect on fasting blood glucose concentrations of the rats (Ibrahim et al., 2019). This was attributed to the duration of feeding especially in young rats where calories may be channelled into growth.

However, it has been shown that feeding adult male Wistar rats a high-fructose diet (10%) for 8 weeks and treating them with 4-OH-Ile 10 days after commencement of the study decreases blood glucose concentrations of the rats (Zhang et al., 2009). This was attributed to the fact that 4-OH-Ile decreases glucose concentrations by accelerating utilization of glucose by tissues and inhibiting hepatic glucose production (Zhang et al., 2009). However, 4-OH-Ile appears to have sexually dimorphic effects between male and female rats as it only decreased the blood glucose concentrations of the males and had no effect on the females. These sexually dimorphic effects should further be investigated.

In pre-diabetic animals, the blood glucose concentrations may be normal, whereas insulin secretion may be abnormal (Leclercq et al., 2007; Simsek et al., 2010). Thus, it is important to also consider blood concentrations of insulin in metabolic studies.

4.3.3 Insulin

Insulin maintains sufficient energy stores and serves as a feedback regulator of plasma glucose (Porte, 2006). In the current study, female rats administered a high-fructose diet with FF had an increased insulin concentration as compared to those on a normal diet. The insulin concentrations of male rats were similar across male groups. This indicates that FF increased insulin concentrations in females on a high-fructose diet. However, insulin concentrations

were not affected by a high-fructose diet alone or a high fructose diet and 4-OH-Ile in female rats. To further assess pancreatic function and whether these findings were biologically significant, HOMA-IR was determined.

4.3.4 Homeostasis model assessment of insulin resistance (HOMA-IR)

The impairment of cells to use insulin is regarded as insulin resistance which is accompanied by an increase in insulin concentrations (Basaranoglu et al., 2015). HOMA-IR is a commonly used computing method of analysing insulin resistance (Radziuk, 2014). The HOMA-IR values of female rats were similar and the HOMA-IR values of all male rats were also similar.

Fructose feeding in rats is generally known to cause insulin resistance and increased insulin concentrations or hyperinsulinemia (Hwang et al., 1987). For instance, a high-fructose diet fed to male adult *Sprague Dawley* rats for 7 days increases insulin concentrations (Tobey et al., 1982). On the contrary, a high-fructose diet (20% fructose) fed to young female and male rats has no effect on insulin concentrations according to Ibrahim et al., 2019 and which is also in accordance with present findings. Tobey et al. (1982) did however use adult rats and fed them for a short period whereas young rats were fed for a longer period of time in this study. Earlier when growth performance was discussed it was noted that fructose-induced metabolic syndrome easily manifests in adults compared to young rats hence, there was no hyperinsulinemia observed in some of the female rats and all male rats and no insulin resistance was observed in the rats (de Moura et al., 2009). Research has shown that the treatment of diabetic adult male Wistar rats with 4-OH-Ile (50mg/kg/day) improves or decreases insulin resistance by increasing peripheral glucose utilization rate (Broca et al., 1999). In the current study, 4-OH-Ile had no effect in both male and female rats and varying outcomes Broca et al. (1999) reported may have been due to the fact that the rats were already diabetic when treatment with 4-OH-Ile started whereas I used fructose to induce metabolic syndrome.

Increased visceral fat is associated with insulin resistance and administration of FF increases β-fat oxidation of visceral fat thus improving insulin sensitivity and resulting in decreased insulin resistance (Jeong and Yoon, 2009). In this study, FF resulted in hyperinsulinemia in females and this suggested that FF caused insulin resistance in females. However, the HOMA-IR computation showed this to not being the case. Even though insulin resistance is one of the main causes of type 2 diabetes, insulin resistance also plays a central role in the

development of non-alcoholic fatty liver diseases (NAFLD) (Armiliato, 2015; Basaranoglu et al., 2015).

4.4 Non-alcoholic fatty liver disease (NAFLD)

NAFLD occurs when there is an accumulation of lipids in the hepatocytes without alcohol abuse or other underlying pathological conditions that might cause steatosis (Castro et al., 2011). NAFLD ranges from simple steatosis to non-alcoholic steatohepatitis (NASH) which includes amongst its characteristics macrovesicular and microvesicular steatosis, inflammation, with or without fibrosis (Benedict & Zhang, 2017). High-fructose diets reportedly increase hepatic triglycerides which are packed into very low density lipoproteins (VLDL) in the liver thus causing hepatic steatosis (Rizkalla, 2010). Hepatic steatosis may also occur due to increased *de novo* lipogenesis, decreased hepatic fatty acid oxidation and increased influx of triglycerides into the liver from extrahepatic mobilization of fatty acid from adipose tissues which can occur in high-fructose feeding (Musso et al., 2009).

In the current study, only female rats developed macrovesicular and microvesicular steatosis. This indicates the ability of fructose to affect the development of steatosis may be sexually dimorphic. For instance, a high-fructose diet (20% fructose) administered to *Sprague Dawley* rats in the neonatal stage and adulthood caused microvesicular and macrovesicular steatosis in both males and females but steatosis was more severe in female rats which are more prone to develop NAFLD compared to male rats on the same treatment (Nyakudya et al., 2018). The findings by Nyakudya et al. (2018) are similar to the present study, despite the differences in study designs in that the development of steatosis in males was not significant however the developed macrovesicular and microvesicular steatosis was significant and severe in female rats. Another study has also shown that feeding 6-week-old male *Sprague Dawley* rats a high-fructose (67% carbohydrate with 98% fructose) diet for 8 weeks caused hepatic steatosis (Nagai et al., 2015). Although *Sprague Dawley* rats were used, Nagai et al. (2015) used older rats and studies have also shown that adult rats are more susceptible to the development of alterations in the hepatic metabolism of lipids as compared to young rats (De Castro et al., 2013). This could account for why Nagai et al. (2015) found steatosis in the older male rats whereas I found no results in my younger male rats. In addition, the differences in effects on sexes may be as a result of sex steroid hormones in male and female rats (Torres et al., 2012).

Conventional drugs have been used to manage NAFLD as the administration of FFhas been reported to decrease hepatic steatosis in experimental animal models (Kostapanos et al., 2013). For instance feeding 6-week-old male *Sprague Dawley* rats a high-fructose diet with FF (30mg/kg/day) for 8 weeks prevented steatosis in the liver however it was not reported whether the steatosis was macrovesicular or microvesicular (Nagai et al., 2015). The expression of PPARα which is largely expressed in the liver is down regulated in rats on a high-fructose diet (Nagai et al., 2015). The decreased activity of PPARα causes a decrease in the β-oxidation of lipids thus causing steatosis in the liver (Lee et al., 2015). However, FF increases protein expression of PPARα by increasing hepatic free fatty acid turnover thus preventing steatosis (Nagai et al., 2015; Kostapanos et al., 2013). The pathogenesis of NAFLD has been proposed to include lipid peroxidation and generation of reactive oxygen species (ROS) or free radicals which cause an inflammatory response followed by the aggregation of hepatic stellate cells thus resulting in fibrosis (Castro et al., 2011). This means that NAFLD can in turn cause inflammation and fibrosis. In the current study, there was no difference on hypertrophy, inflammation and fibrosis scores in female and male rats.

Studies have shown that inflammation activates a stress response which causes lipid accumulation in hepatocytes thus suggesting that inflammation proceeds steatosis (Zunic & Peter, 2018). However, in this study there was no inflammation in the females yet there was steatosis confirming recent literature suggesting that steatosis can be present without inflammation (Zunic & Peter, 2018).

4.5 Circulating lipids

As dyslipidaemia is a primary cause of NAFLD, it necessitated the determination of circulating lipids in rats. Measurement of circulating lipids is important as alterations in concentrations of these lipids are risk factors for poor metabolic health outcomes including the development of ischaemic or coronary heart diseases, type 2 diabetes and insulin resistance (Lehtonen & Viikari, 1978). Dyslipidaemia is a combination of abnormalities including increased triglycerides, decreased high density lipoprotein (HDL) cholesterol which is the ‘good cholesterol’ and increased low density lipoproteins (LDL) cholesterol which is the ‘bad cholesterol’ (Ginsberg et al., 2005). LDL has been regarded as ‘bad’ because it is associated with the development of atherosclerosis through aggregation of foam cells beneath

the endothelium whereas HDL is regarded as ‘good’ because it induces the removal of cholesterol from cells and it’s concentration is inversely proportional to risk for coronary heart disease (Coiweil, 1977; Sacks, 2002; Steinberg et al., 1987).

4.5.1 Triglycerides and cholesterol

The female rats administered fructose and FF had lower triglyceride concentrations and rats administered 4-OH-Ile without fructose feeding had higher triglyceride concentrations compared to female rats on the normal diet. The triglyceride concentrations of male rats were similar across male groups. Fructose increases plasma triglyceride concentrations by increasing *de novo* lipogenesis and decreasing triglyceride clearance (Vos & Lavine, 2013). A 20% fructose diet has been shown to increase plasma triglyceride and cholesterol concentrations in several studies in older rats. For example feeding adult (4 month old) male and female Wistar rats a high-fructose diet (20%) for 8 weeks caused an increase in the plasma triglyceride and cholesterol concentrations (Sandeva et al., 2015). Compared to the present study, that used the same fructose concentration and similar duration but used young rats, the high-fructose diet had no effect on cholesterol and triglyceride concentrations in the two sexes. Apart from age the rodent breed may account for the varying outcomes as there have been studies showing that *Sprague Dawley* rats and Wistar rats respond differently to experimental procedures (Rex et al., 2004). However, a study showed that young rats placed on a high-fructose diet for 8 weeks did not change cholesterol or triglyceride levels (De Castro et al., 2013) which further implicates the impact of age of rats on their responsiveness to high fructose diets. To reiterate, studies which found a difference in lipid levels used adult rats whereas those that reported no differences used young rats. These age-related effects could account for the varying outcomes observed.

4-OH-Ile has been reported to prevent increased triglyceride and cholesterol concentrations in several studies. For example, a study showed that administering 4-OH-Ile (50mg/kg/day) to Golden Syrian dyslipidaemic hamsters for 7 days decreased triglyceride concentrations, lowered LDL cholesterol concentrations and increased HDL cholesterol concentrations as compared to hamsters on the control diet (Narender et al., 2006). Considering that a lower dose of 4-OH-Ile (50mg/kg/day) was effective, we expected a higher dose of 4-OH-Ile (500mg/kg/day) used in this study to be more effective but that was, unexpectedly, not the case. It is notable that hamsters were already dyslipidaemic in the study by Narender et al.

(2006) but dyslipidaemia was induced using a high-fructose diet in the present study. This difference in the disease models between the two studies may account for the different study outcomes. On the contrary to Narender et al. (2006), another study showed that administering 4-OH-Ile without fructose feeding to male Wistar diabetic rats had no effect on the triglyceride concentrations, LDL cholesterol concentrations and HDL cholesterol concentrations (Zafar & Gao, 2016). The current study showed similar results in males whereby rats administered 4-OH-Ile without fructose feeding also had no effect in male rats but caused an increase in triglyceride concentrations of the female rats and possibly resulted in hypertriglyceridemia. This could suggest that the effect of 4-OH-Ile without fructose feeding is sex-specific.

Fenofibrate (FF) has been widely used as a lipid lowering agent as it activates PPARα which is responsible for lipid regulation and lowers triglyceride levels, LDL cholesterol and increases HDL cholesterol concentrations (Keating & Croom, 2007). A study showed that treating monosodium glutamate induced obese male Wistar rats which had hypertriglyceridemia and hypocholesterolaemia with FF (100mg/kg/day), the Fenofibrate decreased triglyceride and total cholesterol concentrations of the rats (Liu et al., 2011). However, rats used in the current study had a normal lipid profile at the start of the study which could account for the varying results. The administration of FF had no effect on triglyceride and cholesterol concentrations of males but decreased the total triglyceride concentrations of female rats fed a high-fructose diet. Other studies have shown that FF has a tendency to decrease triglyceride concentrations, LDL cholesterol and increases HDL cholesterol more effectively in females compared to males. For instance, a study in which adult female mice (which were either overiectomized or sham operated) were fed a high-caloric diet for 8 weeks supplemented with FF, had significantly reduced triglyceride concentrations as compared to the control mice (Yoon et al., 2003). The reduction was more prominent in ovariectiomized rats as compared to sham operated mice suggesting that the absence of oestrogen and progesterone (produced by the ovaries) enhances the lipid lowering ability of agents such as FF(Yoon et al., 2003). Although female rats in this study were not ovariectiomized as the study commenced prepubertally when the sex hormones were low, FF reduced triglycerides in female rats as in the study performed by Yoon et al. (2003). These outcomes confirm the lipid lowering effects of FF in females.

4.6 Visceral organs

When using novel compounds in studies, there is potential for toxicological effects thus it is important to measure the masses of the organs. Measuring the organ-to-body weight ratio (also known as the relative organ weight) is recommended when there are no observed changes on body weight as was the case in the current study (Bailey et al., 2004). Thus, in addition to measuring absolute organ weight, relative organ weights expressed as a percentage were also determined in the current study; however emphasis will be placed on relative organ masses.

There were no differences in most of the measured organs; however FF increased relative liver masses in female rats fed a high-fructose diet. The male rats administered fructose and FF also had the largest relative liver mass compared to all other male rats. Male rats administered fructose and 4-OH-Ile had a larger relative small intestine mass and relative smaller caecal compared to male rats administered the normal diet. The male rats administered fructose and 4-OH-Ile had a greater relative stomach mass compared to male rats administered plain water and 4-OH-Ile and those on the normal diet. FF (30mg/kg/day) has been reported to induce hepatomegaly in 6-week old male *Sprague Dawley* rats on a high-fructose diet for 8 weeks (Nagai et al., 2015). The long-term activation of PPARα receptors which can be caused by long-term feeding of FF has been reported to cause an increase in liver size or hepatomegaly which was the case the current study (Mancini et al., 2001).

The caecum is a site for breaking down of cellulose fibres from plant matter and herbivores usually have enlarged caeca as they consume large amounts of plant matter (Clench & Mathias, 1995). In the current study male rats administered fructose and 4-OH-Ile had the smallest caeca compared to other male rats. This may be due to the male rats consuming relatively more fluid with fructose resulting in ingestion of less solid food material thus decreasing the stimulation of growth of the caecum. This was not observed in females which tended to drink less fructose. Male rats administered 4-OH-Ile had a greater feed intake compared to all male rats. The feed contained crude fibre (45 g/kg) and fibre has been shown to enhance the growth of the small intestine where digestion and absorption of food mainly takes place thus accounts for the enlarged small intestines of male rats (Younoszai et al., 1978). Research has shown a correlation between the size of the stomach and the size of the meals where an increase in meal intake results in a greater stomach size (Santoro, 2012). In

this study, male rats administered 4-OH-Ile had a higher feed intake and also a greater stomach size which confirms the correlation. These outcomes suggest that 4-OH-Ile may be a secondary factor that causes increase of several digestive organs however the sexually dimorphic effects should be further investigated.

4.7 Kidney histomorphometry

Due to their physiological functions, kidneys are vulnerable to pathologic effects of dietary treatments and histologic assessment is the gold standard for assessing kidneys. Hence the kidney histomorphometry was assessed in the current study. In the current study, the renal fibrosis scores were similar in the female rats. Female rats administered fructose only and those administered fructose and FF had smaller renal corpuscle areas as compared to the female rats on the normal diet. The areas of the glomeruli were similar across female groups. The female rats administered fructose and FF had smaller Bowman's capsule space areas compared to females on the normal diet and those administered plain drinking water and 4-OH-Ile and those administered fructose only. There was no difference in the Bowman's capsule space area of female rats administered 4-OH-Ile and those administered the normal diet neither was there a difference between the fructose only group and the normal group. Therefore, suggesting that reduced Bowman's capsule space area which can result in reduced glomerular filtration was due to FF. Recent studies have shown that FF is indeed associated with the development of nephrotoxicity (Attridge et al., 2013). Furthermore, research has shown that approximately 20% of nephrotoxicity, which also ultimately results in reduced glomerular filtration, is caused by drugs thus possibly accounting for the effect of FF in the current study (Kim & Moon, 2012). However due to technical limitations, glomerular filtration was not assessed in the current study.

The fibrosis scores of male rats were similar across male groups. All the male rat groups had smaller renal corpuscle areas compared to the rats on the normal diet. Male rats administered fructose only, fructose with 4-OH-Ile and plain drinking water with 4-OH-Ile had smaller glomeruli area compared to male rats on the normal diet. Male rats administered fructose with 4-OH-Ile and those administered plain drinking water with 4-OH-Ile, had smaller Bowman's capsule space area as compared to male rats administered fructose and FF. It thus seems like males were more prone to the pathological changes from the 4-OH-Ile compared to females. These sexually dimorphic outcomes were not unusual as research has shown that increased

testosterone levels are associated with the development of DN thus accounting for reasons why the male rats may have been more prone than female rats to developing pathological changes from the dietary interventions (Al-Trad et al., 2015).

The administration of fructose to rats has been reported to cause kidney swelling and injury in several rodent studies (Kretowicz et al., 2011). In adult male albino rats, a high-fructose diet (10% fructose in drinking water) administered for 5 weeks caused intra-glomerular fibrosis (Abdel-Kawi et al., 2016). However, in the current study a high-fructose diet had no effect on fibrosis scores of both male and female rats. de Castro et al. (2013) reported diffuse mesangial expansion/glomerulosclerosis in adult fructose-fed rats as compared to young fructose-fed rats (Saleh et al., 2017). The outcomes reported by de Castro et al. (2013) suggest that adults are more prone to developing fibrosis as compared to young rats. In the early pathology of DN in young rats, glomerular membrane thickening and mesangial expansion are absent as they can take up to approximately 9 months to develop (Yang et al., 2018). It is thus possible that in the long term the rats could develop these fibrotic pathologies. However, what were notable in the current study was a reduction in the sizes of the renal corpuscles and the glomeruli rather than an increase in the size of the glomeruli which is usually due to glomerulosclerosis.

Rats start undergoing rapid development of the kidneys around the weaning period which is around day 21 postnatally as was the age of the rats at the beginning of the current study (Seely, 2017). Thus the rats were administered the dietary treatments at a period where the kidneys were not yet fully developed. It is highly likely that the dietary treatments may have stunted their growth and development of the kidney structures thus resulting in the reduced size of the renal corpuscles of the rats. Furthermore the development of the nephrons within the kidney is a very critical period in rodents whereby the "sensitive windows of exposure" are developmental periods when the kidney is vulnerable to drug administration and may result in malformations and nephrotoxicity (Seely, 2017). In the current study FF and 4-OH-Ile were administered during the early post-weaning period which is a "sensitive window of exposure" thus accounting for the negative effects found in the kidneys as a result.

4.8 General health

4.8.1 Red blood cell parameters

Red blood cell indices which include haemoglobin, haematocrit and mean corpuscular haemoglobin (MCH) are used in elucidating aetiology of anaemias thus measuring these indices is important (Wheby, 1980). Haematocrit which is usually expressed as a percentage is the ratio of volume of packed red blood cells to total blood volume (Wennecke, 2004). A low haematocrit percentage indicates a low number of circulating red blood cells or over hydration and a high haematocrit percentage indicates an increase in the number of erythrocytes (Wennecke, 2004). MCH which is the average mass of haemoglobin in each red blood cell decreases when red blood cells are smaller than usual. During erythropoiesis (which is the formation of red blood cells in the bone marrow) nuclear maturation occurs whereby nuclear chromatin condenses, haemoglobin is synthesized in a process called cytoplasmic maturation and cell size is decreased due to water loss and cell division. However defects in nuclear maturation may result in enlarged erythrocytes and thus an increase in the MCH (Wheby, 1980).

Neither high-fructose diet alone nor oral administration of 4-OH-Ile and FF to rats fed a high-fructose diet had an effect on haemoglobin, haematocrit and mean cell haemoglobin (MCH) concentrations of female and male rats. Therefore the dietary treatments most likely had no impact on the bone marrow and erythropoiesis.

CHAPTER 5: CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

5.1 Conclusions

Given the global epidemic of obesity and its implications on healthcare, there is a need for novel interventions to tackle the problem of obesity. The hypothesis for the current study was that 4-OH-Ile would prevent the development of high-fructose diet-induced metabolic dysfunction in growing *Sprague Dawley* rats. However, fructose did not cause a full manifestation of metabolic syndrome as it did not result in abnormalities which are characteristic of metabolic syndrome (e.g. increase in body mass and visceral fat, hypertriglyceridemia, insulin resistance, hyperglycaemia or hypercholesterolemia) in both male and female rats. Despite the absence of no full manifestation of metabolic syndrome, it was observed that NAFLD (microvesicular and macrovesicular steatosis) and diabetic nephropathy (i.e. decreased renal corpuscle size and Bowman's capsule space) developed in high-fructose diet fed female rats. Fructose also caused kidney abnormalities in males by decreasing glomerular sizes. Both NAFLD and diabetic nephropathy place a huge burden on healthcare systems.

4-OH-Ile did not prevent the development of diabetic nephropathy in males on a high-fructose diet as it decreased Bowman's capsule space sizes and glomerular sizes but it prevented the development of NAFLD in males as there was no steatosis, inflammation, hypertrophy or fibrosis. In addition, 4-OH-Ile caused abnormalities in the development of some of the visceral organs in males (i.e. small intestine, stomach, caecum) and may have stunted growth as male rats on a high-fructose diet had a decreased terminal body weight compared to those on a normal diet. Therefore, this showed that administration of chemical agents to young animals with underdeveloped organs may have a deleterious effect and may interfere with normal growth. Therefore phytochemicals need to be fully validated before their medicinal use. There were differences in response between males and females thus confirming the need to investigate both sexes in metabolic studies for validation of phytochemicals from medicinal plants.

In the absence of fructose feeding, 4-OH-Ile caused kidney abnormalities in females by increasing Bowman's capsule space area and in males by decreasing Bowman's capsule space area and glomeruli sizes. In addition, 4-OH-Ile caused microvesicular steatosis and an increase in triglyceride concentrations in female rats which indicates that 4-OH-Ile does have negative effects even in the absence of fructose feeding. Although some of fenugreek's

bioactive compounds namely diosgenin and soluble fibre have previously been shown to have health beneficial properties, 4-OH-Ile in growing males and females, should therefore be administered with caution.

5.2 Limitations and recommendations

Fructose did not induce metabolic syndrome as anticipated and this could have been due to the short feeding period considering the age of the rats or mode of administration. Thus, future studies should consider feeding for a longer period (i.e. > 10 weeks) when using young rats. The dietary treatments had an impact on the nephron structure specifically the Bowman's capsule space which is associated with glomerular filtration (Arif & Nihalani, 2013) however due to technical limitations the glomerular filtration rate was not assessed to further substantiate our findings. Future studies should evaluate glomerular filtration rate in conjunction to measuring Bowman's capsule space area to check whether renal function is impacted by the dietary interventions.

Adiposity can affect fertility (Katib, 2015). In the current study we measured the weights of the epididymal fat but did not assess the sperm quality, semen quality and the fertilizing capacity of the male rats of which dietary interventions at a young age could have an effect on. Thus future studies should further investigate these parameters in males.

The study showed histological changes in liver lipid deposition however due to limited resources, we could not assess the profile of liver lipids. Therefore, future studies should consider analysing the total liver lipids. There is a need for molecular studies to explore underlying mechanisms of these pathologies such as mechanisms involving activation of adenosine monophosphate-activated protein kinase (AMPK) and sterol regulatory binding element protein 1 (SRBEP1) which are involved in development of hepatic steatosis (Luo et al., 2011).

CHAPTER 6: REFERENCES

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APPENDICES

Appendix 1

Clearance certificate from AESC

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2018/03/14/B

APPLICANT: Ms K Motshoane

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: The prophylactic effect of 4-hydroxyisoleucine against high fructose diet induced metabolic dysfunction in growing Sprague Dawley rats

Number and Species

40X 21 day old female and 40X 21 day old male Sprague Dawley Rattus norvegicus

Approval was given for the use of animals for the project described above at an AESC meeting held on 2018/03/27. This approval remains valid until 2020/04/11.
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:

The welfare monitoring will be done when the animals are weighed

Signed: _____ (Chairperson, AESC) Date: 13/04/2018

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: 14/04/2018

cc: Supervisor: Professor K Erlwanger
Director: CAS

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Certificate for modifications and extensions to study approved by the AESC

AREC 2017 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL RESEARCH ETHICS COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Kgomotso Motshoane

b. School and email address: School of Physiology and kgomotsomotshoane2@gmail.com

c. Experiment to be modified / extended

	AREC/AESC NO		
Original AESC number	2018	03	14B
Other M&Ls : change of the use of metformin to the use of fenofibrate as a positive control			1

d. Project Title: The prophylactic effect of 4-hydroxyisoleucine derived from fenugreek seeds against high fructose diet induced metabolic dysfunction in growing rats

		No.	Species
e.	Number and species of animals originally approved:	80	rats
f.	Number of additional animals previously allocated on M&Ls:	0	rats
g.	Total number of animals allocated to the experiment to date:	80	rats
h.	Number of animals used to date:	80	rats

i. Specific modification / extension requested:

To include Ms Chaandanee Bhayroo (1420214) as a co worker on my study.

j. Motivation for modification / extension: Chaandanee Bhayroo (Student no: 1420214) is a Medical Cell Biology Honours student at the School of Anatomical Sciences who has an interest in working on my study. She will be extending the study to focus on the neuropathy section for her Honours degree. She will be using already stored-formalin-preserved tissues (brain and sciatic nerve). She will explore morphological changes in these tissues at microstructural level.

Date: 4/4/2019

Signature:

RECOMMENDATIONS

Addition of Ms Bhayroo (1420214)

Date: 4/4/2019

Signature:

Chairman, AREC

Turn-it-in report