

**EFFECTS OF S-ALLYL-L-CYSTEINE ON
HIGH-FRUCTOSE DIET-INDUCED
NEONATAL METABOLIC
PROGRAMMING IN WISTAR RATS**

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A thesis submitted to the Faculty of Health Sciences, University of Witwatersrand, School of Physiology, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2017

DECLARATION

I, **Busisani Wiseman Lembede**, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

.....

Busisani Wiseman Lembede

Signed on the.....day of.....2017

DEDICATION

In memory of my late grandmother

Thembeni Alizina Lembede

1928 – 2013

RESEARCH OUTPUTS

Conference presentations

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ABSTRACT

The consumption of fructose during the perinatal period programmes for increased susceptibility to developing metabolic derangements immediately in childhood or later in adulthood. S-allyl-cysteine (SAC), a phytochemical constituent of garlic, has antioxidant, antidiabetic and antihyperlipidaemic properties. We hypothesised that neonatal orally administered SAC could protect against the development of high-fructose diet-induced metabolic derangements in both early and adult life.

The study was undertaken in two major experiments. In the first experiment, the potential of neonatal oral administration of SAC to protect against acute metabolic derangements induced by high-fructose diet consumption in early-life was investigated. Sixty-four suckling (male = 32; female = 32) 4-day old Wistar rat pups were randomly allocated to and administered the following treatment regimens daily for 15 days: group I - 10 ml/kg distilled water (DH), group II - 10 ml/kg 20% fructose solution (FS), group III - 150 mg/kg body mass per day SAC, and group IV - (SAC + FS). On postnatal day 21 the pups' blood cholesterol, glucose and triglyceride concentrations were determined. Immediately thereafter the pups were euthanised and tissues collected for analyses. Neonatal orally administered SAC significantly increased ($p < 0.05$) the plasma insulin concentration in male pups. The oral administration of a 20% FS decreased ($p < 0.05$) plasma insulin in the female pups. However, the anti-insulinotropic effect of a 20% FS in female rat pups was attenuated by orally administered SAC. Neonatal orally administered SAC showed insulinotropic effects in male rat pups and protected female rat pups against the anti-insulinotropic effect of a 20% FS.

The second experiment investigated the potential of neonatal orally administered SAC to protect against high-fructose diet-induced metabolic derangements later in adulthood. One hundred and twenty-eight (males = 64; females = 64), 4-day old Wistar rat pups were randomly allocated to and administered treatment regimens as described for the first experiment. On postnatal day 21, the pups were weaned and allowed to grow on a standard rat chow (SRC) until postnatal day 56. The rats from each treatment regimen were then randomly split into two subgroups: one on a standard rat chow (SRC) and plain drinking water and another on SRC and 20% fructose drinking water and then subjected to these treatment regimens for eight weeks after which they were then euthanised and tissues collected for analyses. Neonatal orally administered 20% FS alone, programmed male and female rats to have increased ($p < 0.05$) liver lipid accretion in

adulthood. The neonatal oral administration of SAC attenuated the programming of increased liver lipid accretion in adulthood induced by neonatal orally administered 20% FS. Thus neonatal oral administration of SAC could potentially protect against neonatal fructose consumption programming of fatty-liver related metabolic dysfunctions in adult life.

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LIST OF ABBREVIATIONS

α :	Alpha
β :	Beta
γ :	Gamma
♀:	Female
♂:	Male
AGE:	Aged garlic extracts
ALP:	Alkaline phosphatase
ALT:	Alanine aminotransferase
AMPK:	Adenosine monophosphate activated protein kinase
AOAC:	Association of Official Analytical Chemists
AST:	Aspartate aminotransferase
ANOVA:	Analysis of variance
BMI:	Body mass index
BUN:	Blood urea nitrogen
COX-2:	Cyclooxygenase-2
DH:	Distilled water
DM I:	Type I diabetes mellitus
DM II:	Type II diabetes mellitus
DNA:	Deoxyribonucleic acid
DOHaD	Developmental Origins of Health and Disease
ELISA:	Enzyme-linked immunosorbent assay
FS:	20% fructose solution (w/v)
FW	20% fructose drinking water (w/v)
GIT:	Gastro-intestinal tract
GLUT:	Glucose transporter
GPDH:	Glycerol-3-phosphate dehydrogenase
H and E:	Haematoxylin and eosin
HOMA-IR:	Homeostatic model of insulin resistance
IDF:	International Diabetes Federation
IL:	Interleukin

IM:	Induction mass
LI:	Large intestines
MetS:	Metabolic syndrome
NAFLD:	Non-alcoholic fatty liver disease
NAS:	NAFLD Activity Score
NASH:	non-alcoholic steatohepatitis
NSAID:	Nonsteroidal anti-inflammatory drugs
OGTT:	Oral glucose tolerance test
PDW	Plain drinking water
PND:	Postnatal day
PPAR:	Peroxisome proliferator activated receptor
RG:	Reagent grade
SAC:	S-allyl-cysteine
SF:	S-allyl-cysteine and 20% fructose solution (w/v)
SI:	Small intestine
SRC	Standard rat chow
SREBP-1:	Sterol regulatory element binding protein-1
STZ:	Streptozotocin
TLr:	Relative to tibia length
TBARS:	Thiobarbituric acid reactive substances
TBIL:	Total bilirubin
TCHOL	Total cholesterol
TM:	Terminal mass
TNF:	Tumour necrosis factor
w/v:	weight/volume
WHO:	World Health Organisation
WM:	Weaning mass

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CHAPTER 1: INTRODUCTION AND JUSTIFICATION

1.0 Preview of thesis structure

This thesis is made up of five chapters. Chapter one gives an introduction to the problem of obesity in growing children, an analysis of the research done to date in regard to the problem. It then articulates the justification aims and objectives and hypotheses of the proposed study.

The second chapter provides a critical analysis of the literature pertinent to the study. In summary, the chapter explains and describes the concept of neonatal diet-driven metabolic programming and its effects in early and adult life. Important statistics on the prevalence of obesity and it being a risk factor for metabolic dysfunction/syndrome as manifest by insulin resistance, type II diabetes mellitus, dyslipidaemia and other metabolic derangements is given. Various animal models used in the study of metabolic programming are critiqued. A critical analysis of the merits and demerits of the use of conventional pharmaceutical agents versus the use of ethnomedicine in the management of diet-induced metabolic derangements is presented. A synopsis of the health beneficial effects of S-allyl-cysteine (e.g. antidiabetic, antihyperlipidaemic, antioxidant, hepatoprotective, renoprotective and gastroprotective activity), a phytochemical constituent of garlic, is also presented.

The third chapter starts by giving an introduction and justification, aim and objectives of the first experiment of the study. It gives an account of the interrogation of a critical question: can the oral administration of S-allyl-cysteine during the neonatal growth stage protect against fructose-induced metabolic derangements in early life? The chapter gives a detailed description of the methodology used to answer the question. The chapter then presents the study findings, that is, the effects of oral administration of S-allyl-cysteine on growth performance, markers of metabolic dysfunction (blood parameters and hepatic metabolites), visceral organs and surrogate markers of liver and kidney function of suckling Wistar rats fed a high-fructose diet. A discussion on the significance of the findings and their context in the state of the art are further interrogated in the chapter. The fourth chapter which consists of an introduction, material and methods, results, discussion, and conclusion reports on the findings of the second experiment and of the study. The introduction focuses on a critical analysis of the 'single-hit' or 'double-hit' hypothesis in metabolic programming. The key question that the chapter interrogates is: does neonatal oral administration of S-allyl-cysteine protect against high-fructose diet-induced metabolic derangements in adulthood? The chapter concludes by discussing the pertinent findings.

Chapter five gives a summary of the major findings of the study and draws the attention of the reader to the potential applications of the study findings and key conclusions of the study. Caveats on study limitations are presented and possible future work is proposed.

Chapter six provides the list of references cited in the thesis. Also included in this chapter is the list of appendices.

1.1 Introduction

Worldwide there are 600 million obese adults and 42 million children, under the age of five, who are overweight (World Health Organisation, 2014). Sedentary lifestyles and the consumption of unhealthy western-type diets rich in fats and refined sugars (Pollock *et al.*, 2012), has been ascribed as the main driver of the increase in obesity, non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome (MetS) (Steyn and Temple, 2012).

Evidence from human and animal studies shows that the consumption of fructose-rich diets in adulthood results in increased lipogenesis, visceral obesity, (NAFLD) and (MetS) (Gao *et al.*, 2012; Huynh *et al.*, 2008; Basciano *et al.*, 2005; Steyn *et al.*, 2003; Roglans *et al.*, 2002). These metabolic derangements are not limited to adults. The consumption of a high-fructose diet during early-life (gestation and lactation) increases the risk of developing obesity and insulin resistance in adulthood (Alzamendi *et al.*, 2010; Huynh *et al.*, 2008). Apart from fructose, undernutrition and or overnutrition during gestation and lactation have also been demonstrated to cause epigenetic changes (full definition of epigenetics is provided in chapter 2 under subheading 2.5) that may predispose offspring to develop metabolic derangements during adolescence, and adulthood (Devaskar and Thamocharan, 2007; Léonhardt *et al.*, 2003).

The normal development from the germ cell to multicellular organism is characterised by a sequence of events that are regulated by genetic instructions that are attained at conception (Patel and Srinivasan, 2002). In early critical periods in life, when there is increased plasticity, the organism possesses the capacity to physiologically adapt at molecular, biochemical and cellular levels to suboptimal stimuli (nutritional and environmental stimuli) (Hanley *et al.*, 2010). These physiological adaptations by the organism to suboptimal stimuli in early-life can permanently persist throughout life and modify metabolism and consequently increase the susceptibility or

resistance of developing metabolic derangements in childhood and adulthood (Fernandez-Twinn, and Ozanne, 2010). The phenomenon whereby offspring have an increased or decreased risk of developing systemic dysfunction in early-life (childhood) or in later-life (adulthood) due to earlier gestational and neonatal (the early suckling period) dietary events is termed metabolic programming (Zambrano *et al.*, 2005; Gluckman, and Hanson, 2004). Diet-induced metabolic programming is premised on the “single-hit” and “double-hit” hypotheses. In the single-hit hypothesis, an early-life dietary intervention can result in the onset of metabolic derangements or can serve as an initiator for the onset of metabolic derangement in later life (Tamashiro and Moran, 2010). The “double-hit” hypothesis of metabolic programming is characterised by two dietary interventions; one in early-life (first-hit) which then predisposes to the onset of metabolic derangements following another dietary intervention in later life (the second-hit) (Stewart *et al.*, 2013).

The administration in early-life (gestational and lactation) of substances (pharmacological agents and ethnomedicines) that can prevent or treat obesity, insulin and leptin resistance has been suggested as a feasible intervention for preventing adverse neonatal metabolic programming that predisposes offspring to systemic diseases in adult life (Guilloteau *et al.*, 2009; Vickers *et al.*, 2005). Despite the existence of conventional antiobesity and antidiabetic pharmacological agents, an estimated 70-80% of the global population prefers to use ethnomedicines and specifically plants with medicinal properties to prevent, treat and manage systemic ailments (Sponchiado *et al.*, 2016; Fyhrquist *et al.*, 2002). The preference for the use of plants in ethnomedical practise is based a number of perceived merits: they are regarded as more natural, are claimed to have fewer or reduced side effects and thus are deemed safer than conventional pharmacological agents (Sehgal *et al.*, 2013; Cameron *et al.*, 2011). Ethnomedicinal plant extracts contain an array of biologically and pharmacologically active phytochemicals (organosulphur, polyphenols, vitamins, and minerals) which possess antioxidant, antidiabetic, antiobesity, antimicrobial and cardioprotective properties (Sandhar *et al.*, 2011).

Garlic (*Allium sativum*), a commonly consumed spice, (Eidi *et al.*, 2006) is used in ethnomedicine for the treatment of several systemic diseases (Padiya *et al.*, 2011; Shariyazadeh *et al.*, 2008). The phytochemical constituents of garlic include lipo- and water-soluble organosulphur compounds such as ajoene, diallyl sulphate, alliin, ajoene, allicin and S-allyl-cysteine (Gapter *et al.*, 2008). Garlic’s water soluble sulfur-containing amino acid, S-allyl-

cysteine (SAC), is formed when gamma-glutamyl-cysteine is catabolised by gamma-glutamyltranspeptidase (Lee *et al.*, 2015; Amagase *et al.*, 2001). Studies have shown that SAC possess antimicrobial, antithrombotic, antioxidant, hepatoprotective, neuroprotective, cardioprotective, gastroprotective, antihyperlipidaemic, anti-cancer, anti-inflammatory, antidiabetic and antiobesity properties (Asdaq *et al.*, 2015; Chang *et al.*, 2015; Lee *et al.*, 2015; Choi *et al.*, 2014; Atif *et al.*, 2009; Saravanan *et al.*, 2009; Amagase *et al.*, 2001). S-allyl-cysteine has been demonstrated to attenuate hyperglycaemia, hypertriglyceridaemia, obesity and to improve sensitivity to insulin by tissues in streptozotocin (STZ)-diabetic adult rat models of diabetes mellitus (Saravanan *et al.*, 2009).

1.2 Justification of study

In altricial animals, rats included, neonatal life is characterised by sole dependency on the dam's milk for nourishment. At this stage of life, the animals' organs and physiological systems are immature and there is increased developmental plasticity (Wang *et al.*, 2005). The introduction of or consumption of substances (e.g. fructose and or ethnomedicines) can cause the precocious maturation of organs and metabolic and physiological systems (Jiang *et al.*, 2001; David *et al.*, 1995) and consequently neonatal metabolic programming. Most studies on metabolic derangements have tended to make use of drug-induced models using adult rats yet the increased prevalence of obesity and its (obesity) contribution to a host of metabolic derangements in growing children is well documented (Lobstein *et al.*, 2015; Després *et al.*, 2008). This scenario calls for the use of neonatal animal models that better mimic the problem at hand. Studies on neonatal metabolic programming have largely been indirect whereby the effects of nutritional intervention on pregnant and or nursing dams interrogated have been assessed on the offspring (Comstock *et al.*, 2013; Vickers *et al.*, 2011) with relatively fewer studies on the direct nutritional interventions in neonates, for example, effects of neonatal exposure to high-fructose on the metabolic programming of suckling Wistar rats (Huynh *et al.*, 2008). The majority of studies that have investigated diet-induced metabolic derangements and potential interventions using rat models have mostly generally used male rats (Alzamendi *et al.*, 2010) despite the evidence indicating dissimilarities in physiological development between male and female animals and the existence sex-specific (dependent) responses to interventions (de Sá Couto-Pereira *et al.*, 2016; Ojeda *et al.*, 2016; Pektaş *et al.*, 2015; Rodríguez *et al.*, 2015; Aiken *et al.*, 2013; Mukai *et al.*,

2014; Vickers *et al.*, 2011). Therefore studies that use male and female rats are pivotal to the elucidation of the underlying physiological mechanisms that result in sexually dimorphic metabolic responses in rats and other animals.

The gastrointestinal tract (GIT) is not only vital for the digestion and absorption of nutrients (Zabielski *et al.*, 2008). In early-life, it is involved in regulating the growth and the development of the GIT epithelial lining, immune, neural and metabolic systems (Wiedmeier *et al.*, 2011; Wang *et al.*, 2005). The oral administration of substances during the neonatal growth stage could thus influence digestive and absorptive as well as the regulatory functions of the GIT which could lead to epigenetic changes that then can impact on programming for increased susceptibility to and or resistance to diet-induced metabolic programming (Zabielski *et al.*, 2008; Pérez *et al.*, 2007). Altering nutrient availability and absorption and the development of the GIT can affect overall growth and epigenetic development of the growing animal (Zabielski *et al.*, 2008; Pérez *et al.*, 2007). Neonatal oral administration of a high-fructose diet has been shown to alter the expression of glucose transporters in the GIT and genes that regulate metabolism consequently resulting in adverse metabolic programming (Huynh *et al.*, 2008; David *et al.*, 1995).

Fructose is an inexpensive sugar which is used to sweeten processed foods. Its (fructose) consumption by infants and children has been increasing rapidly in the past decade (Douard and Ferraris, 2013; Basciano *et al.*, 2005). Hence there is a need for research aimed at identifying the potential interventions for high-fructose diet-induced neonatal metabolic programming. Despite the existence of evidence suggesting that early-life administration of antioxidant, antiobesity and antidiabetic substances (pharmaceutical agents and ethnomedicine) has potential to prevent the adverse effects of diet-induced neonatal metabolic programming (Vickers *et al.*, 2005), there still is a dearth of research aimed at identifying probable antioxidant, antiobesity and antidiabetic substances that may prevent the adverse effects of diet-induced neonatal metabolic programming. Furthermore, the high global preference for the use of ethnomedicinal plants demands that researchers need to investigate the potential of traditional ethnomedicines as probable antiobesity and antidiabetic agents (Cameron *et al.*, 2011; Fyhrquist *et al.*, 2002) that may protect against the adverse effects of diet-induced neonatal metabolic programming. Garlic, a globally popular spice and ethnomedicine, contains bioactive phytochemicals such as allicin, alliin and S-allyl-cysteine (SAC) (Amagase, 2006). This multiplicity of phytochemicals with different biological activities (further discussed in chapter 2) introduces many uncontrolled variables for the efficacious

evaluation of the health benefits of garlic. Thus studies need to focus on a single specific purified phytochemicals. The health beneficial biological properties of the garlic-derived phytochemical SAC could potentially be used to mitigate the adverse short- and long-term metabolic programming effects of neonatal high-fructose diet. However, there still exists a dearth in information on the potential of orally administered SAC in suckling rats to protect against the development of adverse effects of neonatal high-fructose diet-induced metabolic programming.

1.3 Aims and objectives

The study was done in two experiments.

i) The aim of the first experiment was to determine the potential of orally administered SAC to protect against the acute development of high-fructose diet-induced metabolic derangements in suckling male and female Wistar rat pups modelling human infants consuming high-fructose diets.

The specific objectives of the first experiment of the study were to determine the effects of orally administered SAC in fructose-administered neonatal Wistar rats on:

- a) growth performance (body mass and linear growth).
- b) non-fasted random blood glucose, triglyceride and cholesterol concentration.
- c) insulin (a hormone that regulates metabolism) concentration and whole-body insulin sensitivity by computing the homoeostasis model assessment of insulin resistance (HOMA-IR index).
- d) liver metabolic substrate (glycogen and lipid) storage.
- e) visceral organ macro- and micro-morphometry.
- f) non-alcoholic fatty liver disease score (NAS).
- g) surrogate markers of liver (alanine aminotransferase, alkaline phosphatase, total bilirubin, globulin, and albumin) and kidney (creatinine and blood urea nitrogen) function and general health.

ii) The aim of the second experiment of the study was to determine the potential of neonatal orally administered SAC to protect against metabolic derangements in adulthood induced by either an 'early single-hit', 'late single-hit' or 'double-hit' high-fructose diet.

The specific objectives of the second experiment of the study were to determine the effects of ‘single-hit’ (early in neonates or late in adults) and or ‘double-hit’ (early in neonates and later in adulthood) by a high-fructose diet in Wistar rats to which SAC was orally administered during the suckling period on:

- a) growth performance (body mass and linear growth).
- b) ability to tolerate an orally administered glucose load.
- c) fasted blood metabolic substrates (glucose, and plasma triglycerides and cholesterol) concentration.
- d) insulin (a hormone that regulates metabolism) concentration.
- e) the HOMA-IR index.
- f) liver lipid content.
- g) visceral organ macro- and micro-morphometry.
- h) non-alcoholic fatty liver disease score (NAS).
- i) surrogate biomarkers of liver (alanine aminotransferase and alkaline phosphatase) and kidney (creatinine and blood urea nitrogen) function.

Research has demonstrated that sex influences the development of the organisms’ physiological systems which consequently impact on how they respond to dietary interventions (Ojeda *et al.*, 2016; Sakuma, 2009; Viveros *et al.*, 2009), hence this study also sought to investigate the presence of potential sexually dimorphic responses to the experimental interventions.

1.4 Hypothesis

1.4.1 Experiment one: hypothesis

H₁: Oral neonatal administration of SAC protects suckling male and female Wistar rat pups against high-fructose diet-induced metabolic derangements in early-life.

H₀: Oral neonatal administration of SAC does not protect suckling male and female Wistar rat pups against high-fructose diet-induced metabolic derangements in early-life.

1.4.2 Experiment two: hypothesis

H₁: Oral neonatal administration of SAC protects male and female Wistar rats against the development in adulthood of metabolic derangements induced by either neonatal or adult “early and late single hit” and or a neonatal followed by adult “double hit” high fructose diet.

H₀: Oral neonatal administration of SAC does not protect adult male and female Wistar rats against the development metabolic derangements induced by either neonatal or adult “single hit” and or a neonatal followed by adult “double hit” high fructose diet.

The next chapter (chapter 2) provides a critical analysis of the literature pertinent to the study. In summary, the chapter explains and describes the concept of neonatal diet-driven metabolic programming and its effects in early and adult life, elucidates current knowledge on obesity, NAFLD, MetS, and diabetes mellitus. The health beneficial effects of garlic and SAC are described.

CHAPTER 2: LITERATURE REVIEW

2.0 Introduction

The increased consumption of energy dense diets and adoption of sedentary lifestyles has led to an upsurge in the prevalence of obesity in children and adults of developed and developing countries (Go *et al.*, 2013; Gupta *et al.*, 2012). Obesity has become a global epidemic (Katzmarzyk *et al.*, 2015). Statistics by the World Health Organisation (WHO) revealed that worldwide more than 1.9 billion adults were overweight and 600 million were obese (WHO, 2014). In 2010 the prevalence of childhood obesity in Africa was estimated to be 8.5% and projected to increase to 12.7% by 2020 (De Onis *et al.*, 2010). Approximately between 5-25% African adult males are obese (WHO, 2014). North America and Australia lead the polls with approximately more than 25% of the adult males being obese (WHO, 2014; Figure 2.1). The WHO estimated that of the adult female population in Southern Africa, greater than 25% are obese and this level of prevalence is comparable to that of adult females in the Americas, Australia and Europe (WHO, 2004; Figure 2.2). Approximately between 5-20% African children are overweight (WHO, 2014). In the Americas region approximately between 5-9.9% of children are overweight; in the Eurasia region an estimated 5-20% of children are overweight (WHO, 2014; Figure 2.3).

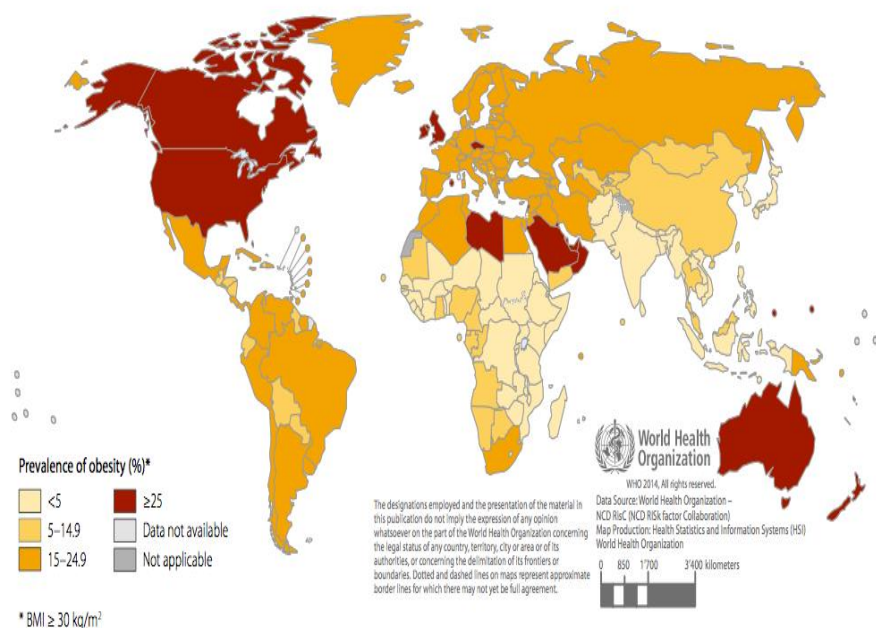


Figure 2.1: A schematic representation of the global obesity epidemic in adult (≥ 18 years of age) males (WHO, 2014).

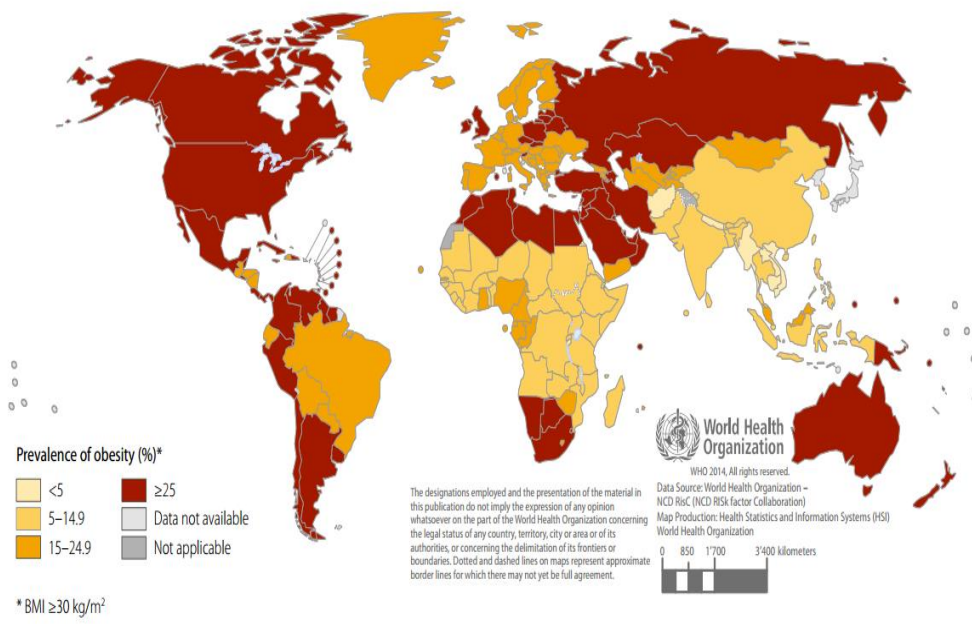


Figure 2.2: A schematic representation of the global obesity epidemic in adult (≥ 18 years of age) females (WHO, 2014).

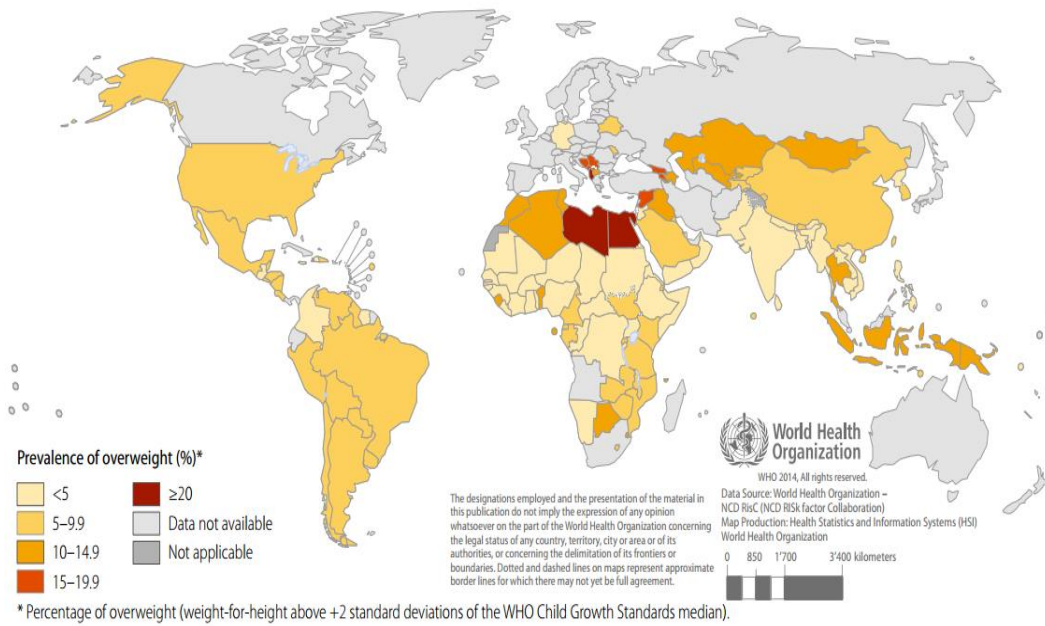


Figure 2.3: A schematic representation of the global prevalence of overweight children (≤ 5 years of age) (WHO, 2014)

The World Health organisation notes that obesity is an increase in fat accumulation that could result in health complications (WHO, 2014). It (obesity) is generally assessed by computing body mass index (BMI) using the equation: $BMI = \text{weight (kg)} / [\text{height (m)}]^2$. The World Health Organisation guidelines state that adults with BMI that is greater than 25 kg/m² are overweight and those with BMI that is greater than 30 kg/m² are obese (WHO, 2014). In children BMI varies with age and sex, hence BMI cut-off points criteria have been developed for children (Cole *et al.*, 2000). Obesity reduces life expectancy and increases the risk of developing cardio-metabolic diseases such as non-alcoholic fatty liver disease (NAFLD), metabolic syndrome (MetS), type II diabetes mellitus (DM II), hypertension and coronary heart disease (Lobstein *et al.*, 2015; Després *et al.*, 2008; Weiss *et al.*, 2004).

2.1 High-fructose diet and obesity

Fructose is an inexpensive monosaccharide naturally found in fruits, vegetables, flowers, and honey (Vos and Lavine, 2013). Commercially fructose is produced via the hydrolysis of vegetables, fruits and corn (Ouyang *et al.*, 2008; Dwivedi and Raniwala, 1980). Fructose is used in the food and beverage industry as a sweetener in carbonated drinks and other sweets (Ouyang *et al.*, 2008). The 10-fold increase in fructose consumption in the past four decades has been associated with the obesity epidemic (Pollock *et al.*, 2012). The absorption and metabolism of fructose differ from that of glucose. Whereas glucose absorption in the jejunum requires glucose transporters 2 (GLUT2) and metabolism triggers phosphofructokinase activity in the liver (Rutledge and Adeli, 2007), the absorption of fructose in the jejunum is facilitated by glucose transporters 5 (GLUT5) and thereafter the fructose is transported to the liver (Sloboda *et al.*, 2014). In the liver, fructose metabolism does not stimulate phosphofructokinase but rather fructokinase activity (Basaranoglu *et al.*, 2013). The latter metabolises it to 3-carbon molecules: glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Vos and Lavine, 2013; Rutledge and Adeli, 2007). These 3-carbon molecules are condensed to form triglycerides and free fatty acids (Miller and Adeli, 2008). The rate limiting enzyme, phosphofructokinase, inhibits the formation of triglycerides from glucose, promotes glycogen formation from glucose and stimulates insulin secretion (Vos *et al.*, 2013). Due to the different pathway through which fructose is metabolised, its consumption results in increased lipogenesis which elicits the development metabolic disturbances such, hyperlipidaemia, hyperglycaemia, glucose intolerance,

hyperinsulinemia, insulin resistance, obesity and ultimately MetS and NAFLD (Mamikutty *et al.*, 2015; Song *et al.*, 2012; Basciano *et al.*, 2005).

2.2 Non-alcoholic fatty liver disease

Obesity has been identified as the main risk factor to increased prevalence of NAFLD in children and adults (Nomura and Yamanouchi, 2012; Roya and Parinaz, 2011). It is estimated that 30% of adults from developed countries have NAFLD (Gaggini *et al.*, 2013). The prevalence of NAFLD in children in the USA aged 15-19 was 17.3% in 2011 (Roya and Parinaz, 2011). Non-alcoholic fatty liver disease, the commonest cause of liver disease in children, is a condition where hepatic lipid accumulation occurs without excessive alcohol consumption (Denzer, 2013). Non-alcoholic fatty liver disease is associated with increased serum activity of alanine aminotransferase (ALT) and amino aspartate (AST) (Ouyang *et al.*, 2008) and increased risk of developing metabolic derangements including insulin resistance, hyperinsulinemia, DM II and MetS (Gaggini *et al.*, 2013). If not managed, NAFLD progresses to non-alcoholic steatohepatitis (NASH), a condition characterised by hepatic inflammation (Kleiner *et al.*, 2005). Non-alcoholic steatohepatitis can result in cirrhosis leading to liver failure (Tappy and Lê, 2012). Histologically, NAFLD can be diagnosed and scored using the NAFLD activity score (NAS) or Brunt's criteria of steatosis (Kleiner *et al.*, 2005; Brunt *et al.*, 1999).

2.3 Metabolic syndrome

While the International Diabetes Federation (IDF) estimates that globally 25% of the adults have MetS, globally approximately 4.2% of adolescent children have MetS (Crespo *et al.*, 2007). Metabolic syndrome (MetS) is defined as a cocktail of risk factors that increase the risk of developing type II diabetes mellitus and cardiovascular complications (Peeters *et al.*, 2014). The cocktail of risk factors for MetS include hyperglycaemia, insulin resistance, general obesity (determined by BMI and waist and hip circumference), visceral obesity, atherogenic dyslipidaemia, and hypertension (Malin *et al.*, 2014). Increased visceral adiposity stimulates the production of pro-inflammatory cytokines: tumour necrosis factor-alpha (TNF- α), interleukin-1 (IL-1), interleukin-6 (IL-6) (Wang *et al.*, 2013). This build-up of adiposity also stimulates the

release of C-reactive proteins (Wang *et al.*, 2013). Pro-inflammatory cytokines suppress the formation of GLUT-4 receptors and inhibit insulin receptor substrate-1 signalling pathway and thus resulting in hyperglycaemia and insulin resistance (Cruz *et al.*, 2013). Hyperglycaemia and insulin resistance result in increased blood non-esterified fatty acids and consequently atherogenic dyslipidaemia (Arunkumar *et al.*, 2012). A combination of elevated blood triglycerides, low-density lipoprotein and decreased high-density lipoprotein cholesterol (HDL-C) is termed atherogenic dyslipidaemia (Peer *et al.*, 2013; Yiyong and Shaobin, 2012). The latter is associated with the development of cardiovascular diseases (Esenabhalu *et al.*, 2015; Navarro-Millán *et al.*, 2015). Atherogenic dyslipidaemia also stimulates increased oxidant production (Navarro-Millán *et al.*, 2015). Increased oxidant production causes oxidative damage to cells, DNA and mitochondria resulting in the inflammation of various organs such as blood vessels and the pancreas (Navarro-Millán *et al.*, 2015; Rashid and Sil, 2015). While oxidant-induced inflammation of the pancreas results in reduced pancreatic β -cell density and subsequently reduced insulin production, inflammation of the blood vessels results in non-compliance and hypertension (Kal *et al.*, 2016; Shen *et al.*, 2013). An increase in inflammatory cytokines combined with atherogenic dyslipidaemia, hyperglycaemia, and insulin resistance can lead to the onset type II diabetes mellitus (DM II) and its associated complications (LaRosa *et al.*, 2013; Matsuda and Shimomurab, 2013).

2.4 Diabetes mellitus

Diabetes mellitus (DM) refers to a group of metabolic conditions that are mainly characterised by hyperglycaemia. Three sub-types of DM exist: type I diabetes mellitus (DM I), type II diabetes mellitus (DM II) and gestational diabetes mellitus. Type I diabetes mellitus results from the failure of pancreatic β -cells to secrete insulin but DM II occurs as a consequence of tissue insensitivity to the stimulatory effects of insulin (Kubo *et al.*, 2016; Hompesch *et al.*, 2015). Gestational DM occurs during pregnancy due to tissues' inability to respond to insulin (Mader *et al.*, 2016). Tissue insensitivity to insulin and or failure of pancreatic β -cells to secrete physiologically adequate quantities of insulin results in the derangement of blood glucose homeostasis. An oral glucose tolerance test (OGTT) assesses the ability to appropriately respond to an oral glucose load. The OGTT is used to screen for diabetes mellitus and pre-diabetes mellitus (Hage *et al.*, 2013). The inability to respond appropriately to a glucose load is associated

with insulin resistance (Fan *et al.*, 2015). Diabetes mellitus is associated with the development of various complications such micro- and macro-vascular damage, organ failure, ischemic heart disease, hypertension, and strokes (Varadarajan *et al.*, 2015).

Research evidence suggests that the GIT can play a significant role in the aetiology of metabolic derangements such as obesity and diabetes mellitus (El Aidy *et al.*, 2015; Kootte *et al.*, 2012; Rubino *et al.*, 2010). The digestive activity in the small intestine accounts for the digestion and absorption of 90% of macro- and micro-nutrients (Grant *et al.*, 2015); the large intestine largely absorbs water and water soluble vitamins produced by colon resident bacteria (Middendorp *et al.*, 2014). Orally consumed substances can alter the GIT's gut microbiota ecosystem which can cause adverse or beneficial effects on the regulation of metabolism and digestion and absorption of nutrients and can subsequently lead to the development of metabolic derangements (Nitin *et al.*, 2016; Moreno-Indias *et al.*, 2015; Kootte *et al.*, 2012). Prenatal and early-postnatal dietary interventions can cause precocious (structural and functional) maturation (including increased lengths and masses) of small and large intestines which translate into accelerated GIT development (He *et al.*, 2013). An increase in the length and masses of the small and large intestines can result in increased digestion and absorption of nutrients (Tatara *et al.*, 2005) which could lead to increased risk and the predisposition to metabolic dysfunctions (De Wit *et al.*, 2008; Nir *et al.*, 1978). Gastrointestinal stimulated satiety has been shown to decrease in obese individuals (Delgado-Aros *et al.*, 2004) and thus further alluding to the importance of the GIT (function and development) in the pathogenesis of metabolic disturbances such as obesity and DM. Nevertheless, the influence of neonatal dietary interventions on the function and development of the GIT and the potential subsequent onset of metabolic derangements such as obesity, NAFLD, MetS and DM II in childhood and adulthood still require further exploration.

2.5 Metabolic programming

Metabolic programming refers to a phenomenon whereby 'deficit' and or 'surplus' nutritional exposure during 'critical' periods of early-life (antenatal and suckling) when there is increased pliability results in epigenetic changes (Dearden *et al.*, 2015). Epigenetic changes refer to alterations that occur in the genome, without altering the genetic code, due to exposure to specific dietary or environment interventions (Fernandez-Twinn *et al.*, 2015). The epigenetic changes in

early-life can result in permanent alteration in molecular, cellular and physiological function that result in ‘thrifty phenotypes’ with increased susceptibility to developing metabolic complications in early (childhood) and or later (adulthood) life (Ong and Ozanne, 2015; Penfold and Ozanne, 2015; Dunn *et al.*, 2009). Dietary interventions in early-life have been shown to determine the resulting phenotype epigenetically by modifying DNA methylation, histones and non-coding RNAs (Wang, 2013; Sohi *et al.*, 2011). For example, the consumption of protein restricted diets, calorie restricted diets, high-energy diets or high-fructose diets during gestation and lactation can down- or up-regulate liver genes involved in the regulation of glucose and lipid metabolism (Burgueño *et al.*, 2013). Alteration of metabolic regulatory genes can then result in the development of obesity, peripheral insulin resistance and hyperleptinemia in the various developmental stages of life (Burgueño *et al.*, 2013; Huynh *et al.*, 2008; Devaskar and Thamocharan, 2007; Palinski and Napoli, 2002). Researchers have developed a variety of animal models for studying nutritional interventions in early-life that result in adverse metabolic programming (Burgueño *et al.*, 2013; Elahi *et al.*, 2009; Desai *et al.*, 2004). Despite the existence of various models for studying metabolic programming, most of them have focused on the effects of early-life (gestation) dietary maternal intervention models of metabolic programming (Oliveira *et al.*, 2015; Bhasin *et al.*, 2009; Desai *et al.*, 2004). Rarely have studies focused on the effects of dietary interventions in neonates during suckling on metabolic programming.

2.5.1 Models of metabolic programming

Numerous models of dietary- and chemically-induced metabolic programming have been established (Oliveira *et al.*, 2015; Burgueño *et al.*, 2013; Bhasin *et al.*, 2009; Elahi *et al.*, 2009; Zhang *et al.*, 2009).

2.5.1.1 Diet-induced models of metabolic programming

Diet-induced models of metabolic programming are established through prenatal and early-postnatal exposure to overnutrition or undernutrition (Oliveira *et al.*, 2015; Burgueño *et al.*, 2013; Bhasin *et al.*, 2009; Elahi *et al.*, 2009). These models are appropriate for investigating the metabolic programming effects of various suboptimal dietary stimuli. Undernutrition models of

metabolic programming are induced by restricting dietary caloric intake or protein intake during prenatal and early-postnatal life (Bhasin *et al.*, 2013; Desai *et al.*, 2004). The overnutrition models of metabolic programming are induced by prenatal and early-postnatal consumption of diets that are high in fat, cholesterol, carbohydrates, sucrose or fructose (Pruis *et al.*, 2014; Plosch *et al.*, 2014; Alzamendi *et al.*, 2010; Huynh *et al.*, 2008).

2.5.1.2 Chemically-induced models of metabolic programming

Chemically-induced models of metabolic programming are developed by inducing maternal diabetes using STZ prior to insemination (Oliveira *et al.*, 2015). Such a chemically-induced model of metabolic programming is suitable for investigating the adverse metabolic programming effects of gestational diabetes on the resultant offspring (Oliveira *et al.*, 2015).

The various models of diet- and or chemical-induced metabolic programming are summarised in the Tables 2.1 to 2.4 below.

Table 2.1: Maternal dietary-insufficiency models of metabolic programming

Species and exposure	Offspring neonatal metabolic disturbances	Offspring adulthood dietary exposure	Offspring adult metabolic disturbance(s)
<ul style="list-style-type: none"> ▪ Sprague-Dawley rats ▪ Calorie restriction during gestation (Desai <i>et al.</i>, 2004) 	<ul style="list-style-type: none"> ▪ ↓ birth weight ▪ Delayed catch-up growth ▪ ↑ ghrelin ▪ ↓ leptin 	<ul style="list-style-type: none"> ▪ Normal rat chow 	<ul style="list-style-type: none"> ▪ ↑ caloric intake ▪ ↑ bodyweight, ▪ ↑ body fat ▪ ↑ leptin ▪ Leptin resistance ▪ Hyperphagia
<ul style="list-style-type: none"> ▪ C57bl/6 mice ▪ Protein restriction during gestation (Ozanne <i>et al.</i>, 2004) 	<ul style="list-style-type: none"> ▪ ↓ birth weight ▪ ↑ catch-up growth/obesity 	<ul style="list-style-type: none"> ▪ Highly palatable diet 	<ul style="list-style-type: none"> ▪ Obesity ▪ ↓ telomere length ▪ ↓ longevity
<ul style="list-style-type: none"> ▪ C57BL/6J mice ▪ Protein restriction during gestation (Bhasin <i>et al.</i>, 2009) 	<ul style="list-style-type: none"> ▪ ↓ essential amino acids ▪ ↓ birth weight 	<ul style="list-style-type: none"> ▪ Normal rat chow 	<ul style="list-style-type: none"> ▪ ↑ catch-up growth/obesity ▪ Obesity ▪ Glucose intolerance

↓ = decreased; ↑ = increased

Table 2.2: Maternal chemical-induced models of metabolic programming

Species and exposure	Offspring adulthood dietary exposure	Offspring adult metabolic disturbance(s)
<ul style="list-style-type: none"> ▪ Wistar rats ▪ Diabetes induced by STZ administered to 5-day old female rats and then fed standard rat chow to adulthood and then mated (Oliveira <i>et al.</i>, 2015) 	<ul style="list-style-type: none"> ▪ Standard rat chow 	<ul style="list-style-type: none"> ▪ ↑ bodyweight, ▪ Obesity ▪ ↑ diameter of adipocytes ▪ ↑ insulin receptor protein ▪ ↑ basal insulin ▪ ↑ insulin-stimulated glucose uptake by adipose tissue ▪ ↑ GLUT4 protein ▪ ↑ acetyl-CoA carboxylase protein

↓ = decreased; ↑ = increased

Table 2.3: Maternal dietary-surplus models of metabolic programming

Species and exposure	Offspring neonatal metabolic disturbances	Offspring adulthood dietary exposure	Offspring adult metabolic disturbance(s)
<ul style="list-style-type: none"> ▪ C57BL/6J mice ▪ Maternal palatable obesogenic diet (16% fat and 33% sugar) during gestation and lactation (Samuelsson <i>et al.</i>, 2008) 	<ul style="list-style-type: none"> ▪ ↑ birth weight ▪ ↑ weaning weight 	<ul style="list-style-type: none"> ▪ Standard rat chow 	<ul style="list-style-type: none"> ▪ ↑ caloric intake ▪ ↑ abdominal fat pad mass ▪ ↑ adipocyte diameter ▪ ↑ plasma triglyceride ▪ Hyperleptinaemia ▪ Hyperinsulinemia ▪ Hypercholesterolemia ▪ Hypertension ▪ Glucose intolerance
<ul style="list-style-type: none"> ▪ Wistar rats; C57BL/6J mice ▪ High-fat diet during gestation and lactation (Burgueño <i>et al.</i>, 2013; Elahi <i>et al.</i>, 2009) 		<ul style="list-style-type: none"> ▪ High-fat diet 	<ul style="list-style-type: none"> ▪ Insulin resistance ▪ ↑ abdominal fat pad ▪ ↑ serum leptin ▪ ↑ hepatic lipid
<ul style="list-style-type: none"> ▪ Wistar rats ▪ High-fat diet 6 weeks before gestation till the end lactation (Férézou-Viala <i>et al.</i>, 2007) 	<ul style="list-style-type: none"> ▪ ↓ weaning body mass 	<ul style="list-style-type: none"> ▪ High-fat diet 	<ul style="list-style-type: none"> ▪ ↑ body mass ▪ Hyperinsulinemia ▪ Hyperleptinaemia ▪ Leptin resistance
<ul style="list-style-type: none"> ▪ LDL receptor-deficient mice ▪ High-fat, high-cholesterol diet 	<ul style="list-style-type: none"> ▪ ↓ birth weight 	<ul style="list-style-type: none"> ▪ Standard rat chow 	<ul style="list-style-type: none"> ▪ ↑ body mass ▪ Aortic atherosclerosis

(Napoli <i>et al.</i> , 2002)			
<ul style="list-style-type: none"> ▪ C57BL/6J mice ▪ High-cholesterol diet (Bhasin <i>et al.</i>, 2009) 		<ul style="list-style-type: none"> ▪ Western diet (high-fat diet with moderate cholesterol) 	<ul style="list-style-type: none"> ▪ Atherosclerosis ▪ Hypercholesterolemia
<ul style="list-style-type: none"> ▪ C57BL/6J mice ▪ Western-style diet during gestation and lactation (Pruis <i>et al.</i>, 2014; Plosch <i>et al.</i>, 2014) 		<ul style="list-style-type: none"> ▪ Western-style diet 	<ul style="list-style-type: none"> ▪ Hepatomegaly ▪ ↑hepatic cholesterol ▪ ↑hepatic triglycerides. ↑lipogenesis ▪ Steatohepatitis ▪ ↑ hepatic inflammation ▪ ↑hepatic transaminases activity
<ul style="list-style-type: none"> ▪ Wistar rats ▪ High-sucrose diet during gestation and lactation (D'Alessandro <i>et al.</i>, 2012) 	<ul style="list-style-type: none"> ▪ ↓birth weight 	<ul style="list-style-type: none"> ▪ High-sucrose diet 	<ul style="list-style-type: none"> ▪ ↑ plasma and hepatic triglycerides ▪ Glucose tolerance ▪ Insulin resistance
<ul style="list-style-type: none"> ▪ Sprague-Dawley rats ▪ Palatable diet during gestation and lactation (Bocarsly <i>et al.</i>, 2012) 			<ul style="list-style-type: none"> ▪ ↑ body mass ▪ ↑ plasma triglycerides ▪ ↑ alcohol consumption
<ul style="list-style-type: none"> ▪ Wistar rats ▪ High-fructose (20% fructose solution) during gestation until 	<ul style="list-style-type: none"> ▪ ↑ birth weights ▪ Hyperglycaemia ▪ ↑ hepatic triglycerides 		

<p>postnatal day 10 (Clayton <i>et al.</i>, 2015; Vickers <i>et al.</i>, 2011)</p>	<ul style="list-style-type: none"> ▪ ↑non-esterified free fatty acid ▪ ↑ALP ▪ ↓ hepatic fructokinase mRNA ▪ ↑ GLUT5 transporter mRNA ▪ ↓ lipoprotein lipase (LPL) RNA ▪ ↓ liver glycogen in males ▪ ↑ liver glycogen in females 		
<ul style="list-style-type: none"> ▪ Sprague-Dawley rats ▪ High-fructose (10% fructose solution) during gestation (Rodríguez <i>et al.</i>, 2013) 	<ul style="list-style-type: none"> ▪ Hypotriglyceridaemia ▪ ↑ hepatic triglyceride and hepatic steatosis ▪ Hyperleptinaemia ▪ ↓ leptin resistance ▪ ↑ lipogenic genes 		
<ul style="list-style-type: none"> ▪ Sprague-Dawley rats ▪ High-fructose diet from 4-weeks of age, during gestation and 		<ul style="list-style-type: none"> ▪ High-fructose diet 	<ul style="list-style-type: none"> ▪ Hypercholesterolemia ▪ ↑ serum triglycerides ▪ ↑ serum free fatty acids

lactation (Ching <i>et al.</i> , 2011)			<ul style="list-style-type: none"> ▪ ↑ hepatic triglycerides ▪ ↑ hepatic free fatty acids ▪ ↑ hepatic lipid
<ul style="list-style-type: none"> ▪ Sprague-Dawley rats ▪ High-fructose, high-fat diet from 4-weeks of age, during gestation and lactation (Chen <i>et al.</i>, 2010) 	<ul style="list-style-type: none"> ▪ ↑ liver mass 	<ul style="list-style-type: none"> ▪ Standard rat diet 	<ul style="list-style-type: none"> ▪ ↑ abdominal fat ▪ Hyperinsulinemia ▪ Insulin resistance
<ul style="list-style-type: none"> ▪ Sprague-Dawley rats ▪ High-fructose diet during lactation (Alzamendi <i>et al.</i>, 2010) 		<ul style="list-style-type: none"> ▪ Standard rat diet 	<ul style="list-style-type: none"> ▪ ↑ body mass and ▪ ↑ adipocyte diameter ▪ ↑ caloric intake ▪ Hyperinsulinemia ▪ Insulin resistance ▪ Hyperleptinaemia ▪ Leptin resistance

↓ = decreased; ↑ = increased

Table 2.4: Neonatal dietary-surplus models of metabolic programming

Species and neonatal exposure	Adulthood dietary exposure	Adulthood metabolic disturbance(s)
<ul style="list-style-type: none"> ▪ Sprague-Dawley rats ▪ High-fat diet from 2 to 21 days old (Haney <i>et al.</i>, 1986) 	<ul style="list-style-type: none"> ▪ Standard rat chow from 21 to 31 days old 	<ul style="list-style-type: none"> ▪ Hyperglycaemia ▪ Hyperinsulinemia
<ul style="list-style-type: none"> ▪ Sprague-Dawley rats ▪ High-carbohydrate diet from 2 to 21 days old (Haney <i>et al.</i>, 1986) 	<ul style="list-style-type: none"> ▪ Standard rat chow from 21 to 31 days old 	<ul style="list-style-type: none"> ▪ Hyperglycaemia ▪ Hyperinsulinemia
<ul style="list-style-type: none"> ▪ Sprague-Dawley rats ▪ High-carbohydrate diet from 4 to 24 days old (Vadlamudi <i>et al.</i>, 1995) 	<ul style="list-style-type: none"> ▪ Standard rat chow from 24 to 60 days old 	<ul style="list-style-type: none"> ▪ Obesity ▪ Pancreatic β-cell hypertrophy ▪ Hyperinsulinemia ▪ Obesity ▪ \uparrowhepatic lipogenic capacity

<ul style="list-style-type: none"> ▪ Sprague-Dawley rats ▪ High-carbohydrate diet from 4 to 24 days old (Hiremagalur <i>et al.</i>, 1993) 	<ul style="list-style-type: none"> ▪ Standard rat chow from 24 to 55 days old 	<ul style="list-style-type: none"> ▪ Obesity ▪ ↑ adipocyte diameter ▪ ↑fatty acid synthase ▪ ↑glucose-6-phosphate dehydrogenase) ▪ Hyperinsulinemia
<ul style="list-style-type: none"> ▪ Sprague-Dawley rats ▪ High-carbohydrate diet from 4 to 24 days old (Petrik <i>et al.</i>, 2001) 	<ul style="list-style-type: none"> ▪ None (rats euthanised when they were 24-days old) 	<ul style="list-style-type: none"> ▪ Endocrine pancreatic apoptosis. ▪ ↓pancreatic islet size and density
<ul style="list-style-type: none"> ▪ Sprague-Dawley rats ▪ High-carbohydrate diet from 4 to 24 days old (Srinivasan <i>et al.</i>, 2008; Srinivasan <i>et al.</i>, 2003a) 	<ul style="list-style-type: none"> ▪ Standard rat chow from 24 to 100 days old 	<ul style="list-style-type: none"> ▪ Hyperphagia ▪ Obesity ▪ Hyperinsulinaemia ▪ ↓hypothalamic insulin and leptin receptor
<ul style="list-style-type: none"> ▪ Sprague-Dawley rats ▪ High-fructose diet (10% fructose solution) from 12 to 19 days old (Huynh <i>et al.</i>, 2008) 	<ul style="list-style-type: none"> ▪ High-fructose diet (65% in Standard rat diet) from 8-11 weeks of age 	<ul style="list-style-type: none"> ▪ ↑ bodyweight ▪ Hyperinsulinemia ▪ ↑ skeletal fatty acid uptake ▪ Increased epididymal fat mass

↓ = decreased; ↑ = increased

The majority of recent studies investigating metabolic programming have focused on the effects of maternal dietary exposure during gestation and lactation on offspring (Oliveira *et al.*, 2015; Bhasin *et al.*, 2009; Desai *et al.*, 2004). However, most of the recent studies have scarcely interrogated the effects of neonatal dietary exposure during suckling on the metabolic programming of adverse short- and long-term metabolic outcomes (Huynh *et al.*, 2008; Petrik *et al.*, 2001). This is despite the availability of data showing that neonatal dietary manipulations, when there is increased developmental plasticity, can programme adverse short- and long-term metabolic outcomes (Huynh *et al.*, 2008; Petrik *et al.*, 2001). The little amount of research on neonatal models of diet-induced metabolic programming has under investigated the potential of ethnomedicinal interventions in preventing or treating the short- and long-term metabolic outcomes induced by metabolic programming.

2.5.2 Interventions for adverse metabolic programming

Numerous studies endorse the administration in early-life (gestational and lactation) of antiobesity and antidiabetic substances (pharmacological agents and ethnomedicines) as possible interventions for preventing, treating and managing neonatal metabolic programming and its adverse outcomes (Desai *et al.*, 2013; Li *et al.*, 2013; Li *et al.*, 2012; Ching *et al.*, 2011; Barbalho *et al.*, 2011; Elahi *et al.*, 2008; Vickers *et al.*, 2005). Li and colleagues (2013) demonstrated that taurine supplementation in the obesogenic diet (high-fat and high-fructose) fed mothers during gestation and lactation attenuated the offspring's hepatic expression of inflammatory cytokines. Oral administration of bitter melon to high-fructose fed litter mother's diet during gestation and lactation attenuated hypercholesterolemia and hypertriglyceridemia and increased antioxidant activity in the liver in the offspring (Ching *et al.*, 2011). In a high-cholesterol maternal diet model, maternal administration of statin during late pregnancy attenuated hypercholesterolemia and hypertension in the resulting offspring (Elahi *et al.*, 2008). Li *et al.* (2012) reported that the administration of green tea stem extract to high-fat diet fed litter mothers during gestation and lactation mitigated insulin resistance in the resulting offspring. "Prevention is better than cure" hence the increased emergence in research devoted to searching for prospective interventions aimed at preventing neonatal metabolic programming and its adverse outcomes such as obesity, NAFLD, MetS and DM II. A wide range of antiobesity and antidiabetic pharmacological agents

that can be used to prevent neonatal metabolic programming and its adverse outcomes exist (Elahi *et al.*, 2008; Vickers *et al.*, 2005). Nevertheless, the majority of the global population consider natural plant-derived ethnomedicines to not cause any adverse side effects to the body (Sehgal *et al.*, 2013; WHO, 2013) and thus prefer the use of natural plant-source-derived ethnomedicines to prevent and or treat various ailments including metabolic disturbances (Sponchiado *et al.*, 2016). The use of ethnomedicines by societies of both the developed and developing countries continues to expand (Burton *et al.*, 2015; WHO, 2013). The expansion in the use of ethnomedicines is largely transmitted through ‘folk referral’ and in most of the cases without and or inadequate scientific validation (Burton *et al.*, 2015). There is, therefore, a critical need to determine, using controlled experiments, the biological, medicinal, and toxicity activity of these ethnomedicines. Such an approach is pivotal for the advancements of ethnomedicinal knowledge systems, their efficacy and safe use.

Garlic is one of the many plants used in ethnomedicine. It contains bioactive phytochemicals whose potential to prevent, treat and or manage negative outcomes of diet-induced metabolic programming still requires further scrutiny.

2.6 Garlic (*Allium sativum*)

The medicinal and nutritional uses of garlic have been documented in Egypt as early as 1500 BC (Rivlin, 2001). Garlic (*Allium sativum*) is commonly used as a spice worldwide (Eidi *et al.*, 2006). Importantly it is also used as an ethnomedicine and over the counter lipid-lowering supplement (Tsai *et al.*, 2012; Hoshino *et al.*, 2001).

2.6.1 Botanical description

Garlic is a bulbous plant that has flat grass-like leaves which are long and narrow (Kamenetsky and Rabinowitch, 2001). Its stalk grows from the bulb and produces spherically arranged white flowers that contain bulbis (seeds) covered by spathae, at the tip (Meyers, 2006). A bulb of garlic is made of bulblet (cloves) enveloped by a white membranous scales (Foster and Duke, 2000).

2.6.2 Taxonomical classification

Allium sativum belongs to the Kingdom Plantae, Class Magnoliopsida, Order Asparagales, Family Amaryllidaceae, Subfamily Allioideae, Genus *Allium*, Species *sativum*.

2.6.3 Ethnomedicinal uses of garlic

The use of various garlic preparations dates back as far 1000 BC (Eidi *et al.*, 2006). Garlic has been used as traditional ethnomedicine to treat gastrointestinal, cardiovascular, infectious, and metabolic diseases and male infertility (Bayan *et al.*, 2014; Rohner *et al.*, 2015; Balamash *et al.*, 2012; Ma *et al.*, 2012; Eidi *et al.*, 2006). The various garlic preparations used as ethnomedicines include garlic powder, garlic oil, aqueous and solvent extracts of raw or dried garlic and aged garlic extracts (AGE). The latter (AGE) are prepared by steeping sliced raw garlic in hydro-ethanol for 20 months and then dried for 72 hours between 68-72°C (Park *et al.*, 2009; Ahmad and Ahmed, 2006).

2.6.4 Pharmacological activities of garlic preparations

The consumption of garlic has the potential to positively or negatively affect GIT morphometry, functionality, and development. Research by Hoshino *et al.* (2001) showed that orally consumed garlic powder caused damage to epithelial cells in the small intestines of male dogs. *In vitro* studies have reported that garlic has antimicrobial effects against fungi and bacteria (Eja *et al.*, 2007; Iwalokun *et al.*, 2004). Microbes are well-known for being pathogenic, however, some form gut flora. Gut flora is important in protection against pathogens, nutrient processing, stimulation and modulation of intestinal immune response and regulation of host fat storage (Filocamo *et al.*, 2012). Garlic has been shown to inhibit adverse gut flora and promote the growth of beneficial gut flora (Filocamo *et al.*, 2012; Ross *et al.*, 2001). Supplementing broiler chicken feed with 0.5% garlic powder (w/w) resulted in improved digestion and absorption of nutrients in the GIT and the growth performance of the birds (Ramiah *et al.*, 2014). The improved

broiler chicken growth performance was ascribed to the garlic-mediated inhibition of microbial pathogens that reduce the efficiency of digestion and absorption (Ramiah *et al.*, 2014). *In vivo* studies done using rats have revealed that in the small intestines garlic inhibits glucose and cholesterol absorption and cholesterol synthesis (Belemkar *et al.*, 2013; Mohammadi *et al.*, 2013) and thus making garlic a potential antidiabetic and antihyperlipidaemic agent.

Garlic extracts attenuate hyperglycaemia, glucose intolerance, dyslipidaemia, proteinuria and reactive oxygen species in streptozotocin (STZ)-induced diabetic rats (Islam and Choi, 2008; Liu *et al.*, 2006; Anwar and Meki, 2003). Thomson *et al.* (2016) noted that intraperitoneally administered aged garlic extracts (AGE) at 300 mg/kg body mass per day or 600 mg/kg body mass per day for 8 weeks attenuated hyperglycaemia, dyslipidaemia, TBARS and oxidant-induced organ (kidney and liver) damage in STZ-diabetic rats. Eidi *et al.* (2006) demonstrated that ethanolic garlic extracts ameliorated hyperglycaemia, hypercholesterolemia, hypertriglyceridaemia and blood urea, uric acid, creatinine, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in STZ-diabetic rats. Aged garlic powder has been reported to increase insulin and reduce fasting blood glucose and glycated haemoglobin concentrations in 3 week-old leptin receptor deficient mice (Seo *et al.*, 2009). Glycation is a common complication associated with in diabetes mellitus which exacerbates oxidant stress, neuro- and retino-pathology and organ damage (Singh *et al.*, 2014). The administration of 3000 mg/kg body mass per day of AGE for 3 months to DM II patients was shown to reduce serum triglycerides and lipid hydroperoxide concentrations (Balamash *et al.*, 2012). Work done by Gómez-Arbeláez *et al.* (2013) demonstrated that the administration of 1.2 g per day of AGE for 2 months to patients diagnosed with MetS increased the concentration of plasma adiponectin proteins, which (adiponectin) regulate glucose and lipid metabolism. In a clinical study, it was noted that DM II patients that were treated with a combination of metformin, a conventional drug used to treat DM and garlic tablets for 24 weeks had significantly greater anti-hyperlipidaemic and anti-hyperglycaemic states compared to DM II patients that received only metformin (Ashraf *et al.*, 2005). Clinical researchers have recommended the addition of garlic to standard DM II treatment regimens as this results in reduced risk of developing diabetic-related cardiovascular complications (Ried *et al.*, 2016; Naser, 2015; Ahmadi *et al.*, 2013; Gómez-Arbeláez *et al.*, 2013; Ried *et al.*, 2013; Sobenin *et al.*, 2008). *In vitro* and *in vivo* research validates and affirms the traditional and clinical use of garlic as an antidiabetic and antiobesity ethnomedicine. Additionally, animal and

human studies have confirmed that garlic reduces cardiovascular complications associated with obesity, MetS and DM II (Ried *et al.*, 2016; Naser, 2015; Ahmad and Ahmed, 2006). The biological mechanisms by which garlic exerts its prophylactic and therapeutic effects are ascribed to it possessing phytochemicals that have antidiabetic, antiobesity, antioxidant and insulinotropic properties (Islam and Choi, 2008; Ahmad and Ahmed, 2006; Ashraf *et al.*, 2005; Eidi *et al.*, 2005; Brace, 2002).

2.6.5 Phytochemical constituents of garlic

The main phytochemical constituents of garlic include water- and lipid- soluble organosulphur compounds such as ajoene, diallyl polysulfides, vinyldithiins, allicin, alliin and S-allyl-cysteine (SAC). Other phytochemical constituents of garlic include phenols (flavonoids, saponins, and tannins) and sterols (Kim *et al.*, 2013; Berginc *et al.*, 2010; Bakri and Douglas, 2005; Borek, 2001).

2.6.5.1 Allicin

Allicin is a lipo-soluble organosulphur that was first isolated and identified in 1944 (Cavallito and Bailey, 1944) and is formed from the lysis of γ -glutamylcystein peptide, alliin (S-allyl-L-cysteine-sulphoxide), by enzymes alliinase (Ankri and Mirelman, 1999). In the past, the majority of studies credited allicin as the primary phytochemical that bestowed on garlic its therapeutic properties (Amagase, 2006). However, recently studies have suggested that allicin is: unstable, responsible for ‘garlic breath,’ can cause adverse effects to the gastrointestinal tract lumen and has low bioavailability (Amagase, 2006; Freeman *et al.*, 1995). These findings have propelled investigations on the therapeutic effects of other organosulphur phytochemicals that are found in garlic such as s-allyl-cysteine (SAC). The focus for the rest of the review will be on SAC which was used in this study.

2.6.5.2 S-allyl-cysteine

The water-soluble, sulphur containing amino acid, SAC is formed from the hydrolysis of γ -glutamyl-S-allyl and is found in abundance in aged garlic (Ray *et al.*, 2011; Krause *et al.*, 2002). S-allyl-cysteine has high bioavailability (103% mice and 98.2% in rats) and is the least toxic of the organosulphur compounds found in garlic (Amagase, 2006; Amagase *et al.*, 2001; Nagae *et al.*, 1994). It (SAC) has been shown to possess various health beneficial biological activities including anticancer, antioxidant, antihyperlipidaemic and antidiabetic activity (Asdaq *et al.*, 2015; Chang *et al.*, 2015; Lee *et al.*, 2015; Choi *et al.*, 2014) and these activities are credited for SAC's protective effects on various organs of the body.

2.6.5.2.1 Antidiabetic activity of S-allyl-cysteine

Hyperglycaemia, insulin resistance, and increased glycation are common features of obesity, NAFLD, MetS and DM II (Saravanan *et al.*, 2010). These features are the key targets for most conventional pharmaceutical drugs and ethnomedicines that are used to prevent, manage or treat obesity, NAFLD, MetS and DM II. In an *in vivo* study, 150 mg/kg SAC attenuated hyperglycaemia in adult STZ-diabetic Wistar rats by increasing insulin, leptin, adiponectin, thyroid hormone concentrations and upregulating the activity of non-enzymatic and enzymatic antioxidants (Saravanan *et al.*, 2013; Saravanan and Ponmurugan, 2013; Saravanan and Ponmurugan, 2012b; Saravanan and Ponmurugan, 2010). Saravanan *et al.* (2009) reported that the administration of S-allyl-cysteine at doses 100 mg/kg and 150 mg/kg body mass for 45 days in STZ-induced diabetic adult Wistar rats ameliorated hyperglycaemia, hypoinsulinaemia and glycosylated haemoglobin. The observed antidiabetic activities of SAC were hypothesised to be due to its ability to inhibit gluconeogenesis by the liver and glucose absorption in the GIT, stimulate insulin secretion by pancreatic β -cells and increase tissue sensitivity to insulin (Iliya *et al.*, 2016a; Saravanan *et al.*, 2009). *In vitro* and *in vivo* research showed that SAC impedes the formation of glycation end products by inhibiting substrates and enzymes that mediate the formation of glycation products (Iliya *et al.*, 2016b; Saravanan *et al.*, 2010; Ahmad *et al.*, 2007).

2.6.5.2.2 Antihyperlipidaemic activity of S-allyl-cysteine

An increased concentration of lipids (triglycerides, cholesterol, and low-density lipoprotein) in blood is termed hyperlipidaemia. Hyperlipidaemia is a common component of metabolic conditions such as NAFLD, MetS and DM II and plays a pivotal role in the pathogenesis of metabolic derangements. In *in vitro* studies, SAC was demonstrated to inhibit cholesterol and free fatty acid synthesis and intracellularly it decreased triacylglycerol concentration, glycerol-3-phosphate dehydrogenase (GPDH) activity, down-regulate adipogenic genes expression and increased adiponectin production in cultured liver cells and 3T3-L1 adipocytes cell lines (Chang *et al.*, 2015; Lui and Yeh, 2001). Findings by Chang and colleagues (2015) demonstrated that SAC inhibited lipogenesis in 3T3-L1 adipocytes by upregulating genes that that promote fatty acid beta-oxidation. *In vitro* work by Hwang and colleagues (2013) revealed that SAC inhibited lipogenesis in steatotic HepG2 cell lines by activating AMP-activated protein kinase (AMPK) and suppressing sterol regulatory element binding protein-1 (SREBP-1) induced lipogenesis. The oral administration of 13.1 mg/kg and 32.76 mg/kg SAC ameliorated hypertriglyceridaemia and hypercholesterolemia and reduced plasma LDL concentrations in adult female Sprague-Dawley rats that were fed high-fat diet for 5 days (Asdaq, 2015). Asdaq (2015) suggested that SAC exerted its therapeutic effects by deactivating 3-hydroxy-3-methylglutaryl-CoA and increasing the activity of lecithin-cholesterol acyltransferase (LCAT) and thus creating conditions that favour lipid hydrolysis. Saravanan and Ponmurugan, (2012a) reported that SAC (150 mg/kg) demonstrated antihyperlipidaemic properties in STZ-diabetic adult Wistar rats. The mechanism by which SAC exerted antihyperlipidaemic properties included increasing insulin secretion and sensitivity and thus increasing cholesterol clearance and decreasing LDL (Saravanan and Ponmurugan, 2012a). The administration of SAC (0.45% in diet) was observed to attenuate hypertriglyceridaemia and hypercholesterolemia in non-alcoholic fatty liver diseased Otsuka Long-Evans Tokushima male rats by upregulating hepatic proliferator-activated receptors alpha and gamma (Takemura *et al.*, 2013).

2.6.5.2.3 Antioxidant activity of S-allyl-cysteine

Oxidative stress, an imbalance between free radical production and the body's capacity to eliminate their adverse effects, is a common feature in various metabolic disturbances such as obesity, NAFLD, MetS and DM II and can result in tissue, organ damage and disturbance of various homeostatic systems (Schaffer *et al.*, 2012). Orally administered SAC attenuates elevated blood markers of antioxidant stress: malondialdehyde, glutathione peroxidase enzyme activity, total glutathione and oxidised glutathione in serum and thiobarbituric acid reactive species in adult female Sprague-Dawley rats that were fed high-fat diet for 5 days (Asdaq, 2015) suggesting that SAC could potentially be used as intervention for protecting against oxidant-induced cellular damage and its negative consequent complications.

2.6.5.2.4 Cardioprotective activity of S-allyl-cysteine

Obesity, NAFLD, MetS and DM II are associated with the development of cardiovascular diseases such as hypertension and coronary heart disease (Poirier *et al.*, 2006). S-allyl-cysteine has been demonstrated to protect cardiomyocytes against myocardial infarction and oxidant-induced mitochondrial injury in adult male Sprague-Dawley rats (Wang *et al.*, 2010; Chuah *et al.*, 2007). It (SAC) exerts these protective effects by reducing of hydrogen sulphide (H₂S) and increasing the activities of antioxidant enzyme superoxide dismutase (Wang *et al.*, 2010).

2.6.5.2.5 Hepatoprotective activity of S-allyl-cysteine

Metabolic syndrome and diabetes mellitus are associated with hyperlipidaemia induced liver cell damage (Cordeiro *et al.*, 2015). Increased serum activity of liver enzymes [aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP)] is indicative of liver damage (Asdaq, 2015). Studies by Saravanan *et al.* (2009) and Asdaq (2015) reported that the oral administration of SAC attenuated increased serum activity of liver enzymes (AST, ALT, and ALP) in STZ-diabetic adult Wistar rats and high-fat diet fed Sprague-Dawley rats, respectively.

2.6.5.2.6 Renoprotective activity of S-allyl-cysteine

Renal failure is one of the most common complications of metabolic syndrome and DM II (Gluba *et al.*, 2013). It (renal failure) can result in elevated blood urea and creatinine concentrations (Cavalcanti *et al.*, 2006). Orally administered SAC has been reported to prevent kidney inflammation and mitigate elevation of blood urea and creatinine concentrations in STZ-diabetic adult Wistar rats (Mong and Yin, 2012; Saravanan *et al.*, 2009). The renoprotective effects of SAC are due to its ability to upregulate peroxisome proliferator-activated receptors (PPAR) - α and - γ and increase antioxidant activity in the kidneys (Mong and Yin, 2012; Saravanan *et al.*, 2009).

2.6.5.2.7 Gastroprotective activity of S-allyl-cysteine

Ethnomedicines contain phytochemicals that have been reported to have potential to adversely affect GIT micro- and macro morphometry (Metges *et al.*, 2011; Tatara *et al.*, 2005). Orally administration of SAC (3, 5, or 10 mg/kg body mass per day) protected the GIT against ethanol- and nonsteroidal anti-inflammatory drugs (NSAIDs)- induced acute gastric mucosal damage by attenuating chemical induced release of cyclooxygenase-2 (COX-2) in adult Sprague-Dawley rats (Choi *et al.*, 2015; Choi *et al.*, 2014; Park *et al.*, 2014). The orally administered SAC protected the GIT by promoting mucus secretion, increasing antioxidant activity of heme oxygenase-1 and suppressing COX-2 (Choi *et al.*, 2014; Park *et al.*, 2014).

2.6.5.2.8 Neuroprotective activity of S-allyl-cysteine

Peripheral neuropathy, a common complication of diabetes mellitus, occurs due to hyperglycaemia-induced neurone damage and is characterised by pain and numbness in the extremities (Van Dam *et al.*, 2013). Ito *et al.* (2003) reported that SAC protected neurones in the hippocampus against amyloid- β -induced neurotoxicity. *In vitro* and *in vivo* studies have reported that SAC protects neurons against ischemic-induced damage due to its free radical scavenging activity and ability to inhibit the extracellular signal-regulated kinases (ERK) signalling pathways

(Shi *et al.*, 2015; Imai *et al.*, 2014; Atif *et al.*, 2009 and Kim *et al.*, 2006). Due to its neuroprotective (protection against synaptic degeneration and neuroinflammatory pathways) properties, SAC has been recommended as a feasible intervention for Alzheimer's disease (Ray *et al.*, 2011).

2.6.5.2.9 Anticancer activity of S-allyl-cysteine

Obesity has been linked with the development of various cancers including oral cancer (Petti, 2009; Calle and Kaaks, 2004; Calle *et al.*, 2003). *In vitro* work has demonstrated that SAC anticancer activity against human oral squamous cancer CAL-27 cells, breast cancer cell and human ovarian cancer cell (Xu *et al.*, 2014; Tang *et al.*, 2009; Gapter *et al.*, 2008). S-allyl-cysteine exerts its anticancer effects by inhibiting tumour cell proliferation and metastasis and downregulating tumour pro-malignant proteins (Xu *et al.*, 2014; Tang *et al.*, 2009; Gapter *et al.*, 2008).

2.6.5.2.10 Toxicity of S-allyl-cysteine

The use of phytochemicals derived from ethnomedicinally important plants has the potential of eliciting adverse toxic effects. Work by Kodera and colleagues (2002) showed that in mice and rats, oral (po) and intraperitoneal (ip) administration of SAC (2100 to 15000 mg/kg) exhibited minimal toxicity with lethal doses (LD₅₀) > 8.8 g/kg for po and > 3.2 g/kg for ip. In a subacute study orally (po) and intraperitoneally (ip) administered SAC (250, 500, 1000, and 2000 mg/kg body mass) was reported to cause dose-dependent reversible subacute toxicity at doses > 500 mg/kg (Kodera *et al.*, 2002). The subacute toxicity was characterised by decreased urinal urobilinogen, increased serum glucose, total cholesterol, and total protein concentrations, pancreatic atrophy, and hypertrophy of the liver and kidneys (Kodera *et al.*, 2002).

The next chapter (chapter 3) provides further specific background literature and a justification for the first experiment which (experiment) sought to determine the potential of neonatal orally administered SAC to protect against adverse metabolic derangements induced by an orally

administered high-fructose diet (20% fructose solution) in suckling Wistar rat pups modelling human infants fed high-fructose diets.

**CHAPTER 3: EFFECT OF ORALLY
ADMINISTERED S-ALLYL-CYSTEINE
AND FRUCTOSE IN NEONATAL RATS**

3.0 Introduction

The prevalence of obesity in South African children was reported to be at 4.2% in 2002 (Zeelie *et al.*, 2010) and it is estimated that it will increase to 22.8% by the year 2020 (Toriola *et al.*, 2012). The projected increase in the prevalence of childhood obesity is due to the increase in sedentary lifestyles and poor dietary choices such as the consumption of foods that are rich in fructose (Go *et al.*, 2013) and also in fat (Evans *et al.*, 2014). These poor dietary choices and the practice of sedentary lifestyles is a characteristic feature of communities in both the developed and developing world (Gupta *et al.*, 2012). Obesity has been recognised as the leading contributor to the increase in the prevalence of non-alcoholic fatty liver disease (NAFLD) in children (Mager *et al.*, 2015). Non-alcoholic fatty liver disease, a condition characterised by the excessive accumulation of lipids in the liver (Alisi and Nobili, 2014), is considered an early stage condition in the development of metabolic syndrome (MetS) (Mager *et al.*, 2015). Metabolic syndrome is a multifactorial disorder that involves the interaction of various conditions such as hyperglycaemia, insulin resistance, abdominal obesity, atherogenic dyslipidaemia and hypertension (Malin *et al.*, 2014). The manifestation of metabolic derangements such as obesity, NAFLD, and MetS during periods of increased plasticity (gestational and or lactation) can result in the development of adverse neonatal metabolic programming (Huynh *et al.*, 2008).

Neonatal metabolic programming refers to the phenomenon whereby dietary manipulation during early-life (gestation and lactation) causes epigenetic changes that result in an organism developing increased susceptibility or resistance to disease in later life (Guilloteau *et al.*, 2009). The feeding of a high-fructose diet to suckling rat pups has been demonstrated to cause obesity, NAFLD and MetS in early-life and increased susceptibility to develop cardio-metabolic complications in adult life (Jegatheesan *et al.*, 2015; Huynh *et al.*, 2008). Early-life (gestational and or lactation) administration of antidiabetic and antiobesity pharmacological agents has been advocated as a feasible intervention for preventing the diet-induced neonatal metabolic programming that predisposes offspring to systemic diseases later in adult life (Guilloteau *et al.*, 2009; Vickers *et al.*, 2005). In altricial species, the oral administration of antidiabetic substances in neonatal life when the GIT is undeveloped has the potential to alter the GIT microbiota ecosystem (Buddington *et al.*, 2011). This alteration can consequently result in altered GIT development, structure, and functionality (Rist *et al.*, 2013; Schumann *et al.*, 2005). In neonatal

life, the GIT plays an elementary role in nutrition partitioning, gene expression and development of various physiological systems including the endocrine system (Rist *et al.*, 2013; Buddington *et al.*, 2011; Srinivasan *et al.*, 2003b). Thus altering GIT development, structure and functionality in neonatal life can potentially result in altered growth, development and the programming of short- and long-term adverse metabolic outcomes.

An array of conventional pharmacological agents that are used to prevent and or treat obesity, NAFLD, MetS and DM II exist (Padiya *et al.*, 2011; Kraja *et al.*, 2010). However, 80% of the global population still depends on ethnomedicines to treat ailments hence the observed global growth in the ethnomedicinal business (Sponchiado *et al.*, 2016). Ethnomedicines are naturally occurring and are thus considered to be in harmony with the body and to have a minimal potential for causing adverse effects (Sehgal *et al.*, 2013). Research envisioned at cultivating information about ethnomedicines is pivotal at driving the growth of indigenous knowledge systems (Sponchiado *et al.*, 2016). Ethnomedicines contain numerous health beneficial bioactive phytochemicals that contribute to their medicinal properties (Sandhar *et al.*, 2011).

Garlic, *Allium sativum*, is globally used as a spice and as an ethnomedicine for treating systemic disorders (Banerjee and Maulik, 2002). It has been reported to possess anticancer, antidiabetic, antimicrobial, immunoboosting, hepatoprotective, antifibrinolytic and cardioprotective effects (Santhosha *et al.*, 2013). The phytochemical constituents that give garlic its medicinal properties include organo-soluble compounds, alliin, allicin, diallyl disulphide, trans-ajoen and water-soluble organosulphur peptide S-allyl cysteine (SAC). S-allyl cysteine, found in abundance in aged garlic extracts (AGE), has been shown *in vitro* and *in vivo* to possess hepatoprotective, antioxidant, antiobesity and antidiabetic properties (Saravanan *et al.*, 2009; Lin *et al.*, 2008; Ahmad *et al.*, 2007). Research has demonstrated that SAC attenuates oxidative stress, hyperglycaemia and dyslipidaemia in adult STZ-diabetic and high-fat diet rat models by increasing lipid breakdown, insulin sensitivity and insulin secretion (Saravanan and Ponnuragan, 2010; Lin *et al.*, 2008; Ahmad *et al.*, 2007). The health beneficial medicinal properties of SAC render it a plausible candidate for preventing high-fructose diet-induced metabolic disturbances in early-life. Thus this study sought to determine if the neonatal oral administration of SAC could protect suckling Wistar rats against the development of high-fructose diet-induced metabolic derangements by specifically determining SAC's effects on growth performance, markers of

metabolic dysfunction (blood glucose, triglyceride, cholesterol, insulin concentration and hepatic glycogen and lipid content), viscera morphometry and surrogate markers of liver (alanine aminotransferase, alkaline phosphatase, total bilirubin, globulin, and albumin) and kidney (creatinine and blood urea nitrogen) function.

Hypothesis

H₁: Oral neonatal administration of SAC protects suckling male and female Wistar rat pups against high-fructose diet-induced metabolic derangements in early-life.

H₀: Oral neonatal administration of SAC does not protect suckling male and female Wistar rat pups against high-fructose diet-induced metabolic derangements in early-life.

3.1 Materials and methods

3.1.1 Source of S-allyl-cysteine

Reagent grade (RG), S-allyl-cysteine was procured from ChromaDex (California, Irvine, USA).

3.1.2. Ethical clearance for the study

Ethical clearance for the study was granted by the Animal Ethics Screening Committee (AESC) of the University of Witwatersrand. The AESC clearance number was 2015/07/B. Copies of the certificate (and further modifications to the study) are included as appendices (see Appendices 1 and 2).

3.1.3 Animals, feeding, and housing

Sixty-four, four-day-old male (n = 32) and female (n = 32) suckling Wistar rat pups from first-time breeders were used in the study. The rat litters were housed in the Central Animal Services unit with their respective dams in Perspex cages with stainless steel mesh lids and were allowed

ad libitum suckling of their mother's milk throughout the experiment. Bedding made up of clean wood shavings was provided and changed twice per week. Room temperature was maintained at $24 \pm 2^{\circ}\text{C}$ with a 12-h light/dark cycle (lights on from 07:00 h to 19:00 h). The pups were acclimatised for two days (postnatal days 4 and 5) prior to the commencement of the experiment.

3.1.4 Experimental design

The pups were randomly allocated (on postnatal day 6) to and administered one of the following treatment regimens: group I - 10 ml/kg body mass per day distilled water (DH), group II - 10 ml/kg body mass per day 20% fructose solution (w/v) [FS], group III - 150 mg/kg body mass per day of S-allyl-cysteine (SAC) dissolved in distilled water, group IV - 150 mg/kg body mass per day of SAC dissolved in distilled water and 10 ml/kg body mass per day of 20% fructose solution (w/v) [SAC + FS]. The respective treatment regimens were orally administered as a single bolus daily to the respective rat pups for 15 days (postnatal day 6 to 20) using a 22 G plastic cannula (Vasocan® Braunüle®, B. Braun Medical (Pty) Ltd, Northriding, Johannesburg, South Africa). The random allocation was such that there were 8 male and 8 female pups per treatment regimen.

3.1.5 Body mass measurement

The pups were weighed daily using an electronic scale (Snowrex Electronic Scale, Clover Scales, Johannesburg) to monitor growth performance. The body mass measurements also permitted for the maintenance of a constant dosage of the treatment regimens relative to body mass over the 15 day treatment period.

3.1.6 Terminal procedures

3.1.6.1 Determination of terminal body mass and non-fasted blood metabolites

On postnatal day 21, the rat pups' terminal body masses were measured using an electronic scale (Snowrex Electronic Scale, Clover Scales, Johannesburg). Thereafter a drop of blood was

obtained from the tail vein via pinprick and used to determine non-fasted blood glucose using a calibrated Contour plus glucometer (Bayer, Isando, South Africa) as per the manufacturer's instructions. The rat pups were not fasted overnight prior to termination as they were still young (preweaning) and dependent on their dams' milk for nourishment. Moreover an overnight fast would have required maternal separation which has been shown to cause stress and altered metabolic function (Zalosnik *et al.*, 2014; da Silva Lima *et al.*, 2011). The pups were then euthanised by intraperitoneal administration of 100 mg/kg body mass of sodium pentobarbital (Eutha-naze, Bayer, Johannesburg, South Africa). Following euthanasia, blood was collected via cardiac puncture using 21 G needles and 5 ml syringes into 4 ml heparinised blood collection tubes (Becton Dickinson VACUTAINER Systems Europe, Meylan Cedex, France). A drop of the blood was then used to determine the concentration of blood triglycerides and cholesterol using calibrated Accutrend triglyceride and cholesterol meters (Roche, Mannheim, Germany) according to the manufacturer's instructions. The remaining heparinised blood samples were spun for 15 min at 5000 g at 22°C in a Senova NovaFuge centrifuge (Senova Labtech, Shanghai, China). The plasma was then collected into microtubes and stored at -20°C for later determination of plasma insulin concentration and surrogate markers of liver and kidney function.

3.1.6.2 Determination of visceral organ morphometry

Following blood collection, the abdomen was cut open by a midline incision. The liver, small and large intestines were carefully dissected out. The small and large intestines were gently stretched out on a dissection board and their lengths were measured using a ruler mounted on the dissection board. The contents of the small and large intestines were gently emptied after which the visceral organs were weighed on an electronic balance (Presica 310M, Presica Instruments AG, Switzerland). A sample of the liver was stored at -20°C for determination of liver glycogen and lipid content. Liver samples for histology analysis were preserved in 10% phosphate buffered formalin.

3.1.6.3 Determination of liver histo-morphometry

The preserved liver samples were embedded in paraffin wax, sectioned, and then stained with haematoxylin and eosin on a glass slide and covered with a glass coverslip (Reyes-Gordillo *et al.*, 2007). To assess hepatocellular changes, specifically hepatocyte size and cell numbers within a linear field (100µm) the slides were viewed under a light microscope using an eyepiece micrometer (Reichert®, Austria) at high power magnification of 400 X. Photographs of the slides sections were captured using a camera mounted onto the microscope. The NAFLD Activity Score (NAS), which is a semi-quantitative grading and scoring system, was used to grade the ballooning, lobular inflammation, and hepatic steatosis (Kleiner *et al.*, 2005). A detailed description of the NAS criteria is attached in appendix 3.

3.1.7 Liver glycogen and lipid content determination

Liver glycogen content was determined by using a method of indirect acid hydrolysis of glycogen to glucose as described by Passonneau and Lauderdale, (1974). In summary, 0.1 g of liver sample was placed in 1 ml of 0.03 M HCl and thereafter homogenised using an Ultraturrax homogeniser (Janke and Kunkel, Ika-Werk, Germany). Then 1ml of 1 M HCl was added prior to the mixture being boiled for 2 hours in a water bath. Thereafter the sample was allowed to cool following which it was then neutralised by adding 1 ml of 1 M NaOH. The glucose concentration of the hydrosylate was then determined using an Accu-Chek Active glucometer (Roche, Germany).

The total liver lipid content was determined by the soxhlet method (ether extract) of extraction as described by AOAC (2005; method number 920.39).

3.1.8 Determination of plasma insulin concentration and computation of HOMA-IR index

Plasma insulin concentration was determined by enzyme-linked immunosorbent assay (ELISA) using a rat insulin kit [Elabscience ®, Rat INS (Insulin) ELISA kit, Wuhan, Hubei Province, China] according to the manufacturer's instructions. A detailed description of the protocol used is attached in appendix 4. Briefly, the assay used a sandwich enzyme immunoassay method

utilising a monoclonal antibody specific for rat insulin with a sensitivity range of 3.125-200 ng/mL. Absorbencies were read at 450 nm using a microplate reader (Multiskan Ascent, Lab system, model n° 354, Helsinki, Finland). A standard curve was constructed using calibrator concentrations. The concentrations of insulin in the samples were then determined from the constructed standard curve.

Whole-body insulin sensitivity was then computed using the homoeostasis model assessment of insulin resistance as illustrated in the equation below:

$$\text{HOMA-IR} = [\text{fasting insulin concentration (ng/mL)} \times \text{fasting glucose concentration (mg/dL)}] / 405$$

(Matthews *et al.*, 1985).

To convert mmol/L fasting glucose concentration to mg/dL, mmol/L was multiplied by 18.01.

3.1.9 Determination of linear growth

The right hind leg was removed from each of the carcasses, defleshed and then the femur and tibia were separated. The bones were then dried to a constant mass in an oven (Salvis®, Salvis Lab, Switzerland) at 40°C for 7 days. The dry tibiae masses were then determined using an electronic scale (Presica 310M, Presica Instruments AG, Switzerland). The lengths of the tibiae were measured between tibia head and medial malleolus using digital vernier callipers [Major Tech (Pty) Ltd, KTV 150 digital calliper, Elandsfontein, South Africa]. The density of the tibiae were approximated by computing the Seedor index (Seedor *et al.*, 1991):

Seedor index (mg/mm) = dry mass of bone (mg) /bone length (mm).

Radiographs of the tibiae were taken using a Shimadzu MVX 200 X-ray machine (Shimadzu Corp, Kyoto, Japan). Briefly, the bones were placed on the 0.71 mA photographic plate at a distance of 1 metre from the X-ray light source with settings of 5.3 kVp.

3.1.10 Determination of biochemical surrogate markers of liver and kidney health

The plasma activities of ALT and ALP and concentration of albumin, globulin, total protein, total bilirubin, blood urea nitrogen and creatinine of the rat pups were determined using a colorimetric-based clinical chemistry analyser (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., USA) as per the manufacturer's instructions. Briefly, each stored plasma sample was thawed and allowed to warm to room temperature, and then gently inverted to mix the contents. The plasma sample was then placed into the analyser which automatically drew up 150 µL of the plasma and dispensed 10 µL onto each of the pre-loaded disks and the sample was then analysed and a print out of results provided.

3.1.11 Statistical analysis

Parametric data are expressed as mean ± SD and non-parametric data are expressed as median and range (min, max). The data was analysed using GraphPad Prism 5 software (Graph-Pad Software Inc., San Diego, CA, USA). Statistical significance was considered when $p \leq 0.05$. Body mass data within groups was analysed using a repeated measures analysis of variance (ANOVA). A one-way ANOVA was used to analyse other multiple group data followed by mean comparison using a Bonferroni *post hoc* test. The Kruskal-Wallis test (non-parametric one-way ANOVA) was used to analyse non-alcoholic fatty liver disease score (NAS) multiple group data followed by Dunns *post hoc* test to compare medians.

The model used for the analysis of variance for daily body masses was:

$$Y_{ijk} = \mu + T_i + B_j + C_k + e_{ijk}; \text{ where;}$$

Y_{ijk} = daily body mass

μ = overall mean common to all observations

T_i = effect of treatment regimen ($n = 1,2,..4$)

B_j = fixed effect of individual rat (1,2,3.....32)

C_k = fixed effect of sampling day on body mass (= 1,2.....16)

e_{ijk} = residual random error

The model used for variables determined at study termination was:

$Y_{ijk} = \mu + T_i + B_j + e_{ijk}$; where;

Y_{ijk} = response variable of interest

μ = overall mean to all observations

T_i = effect of treatment (n = 1,2,..4)

B_j = fixed effect of individual rat (1,2,3.....32)

e_{ijk} = residual random error

3.2 Results

3.2.1 Growth performance

3.2.1.1 *Body mass*

Figure 3.1 below shows induction and terminal body masses of male rat pups.

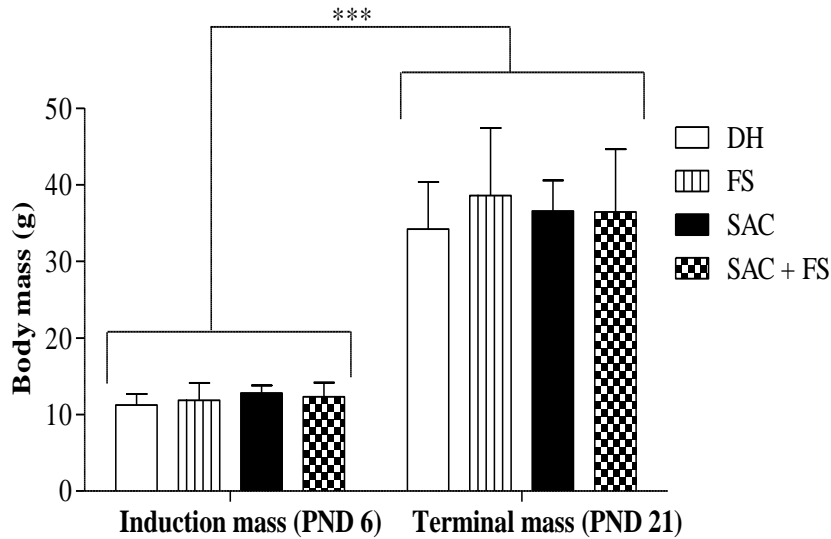


Figure 3.1: Effect of S-allyl-cysteine on terminal body masses of the male rat pups fed a high-fructose diet.

*** $p < 0.0001$. Induction body masses of the rats were similar across treatment regimens. Across treatment regimens, the rat pups significantly grew ($p < 0.0001$). However terminal body masses of the rats were similar across treatment regimens. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v); PND = postnatal day. Data presented as mean \pm SD; n = 8 per treatment.

Although the male rat pups across treatment regimens grew significantly ($p < 0.0001$) over the experimental period (Figure 3.1), the induction and terminal body masses of the male rat pups across treatment regimens were similar (Figure 3.1).

Figure 3.2 below shows induction and terminal body masses of female rat pups.

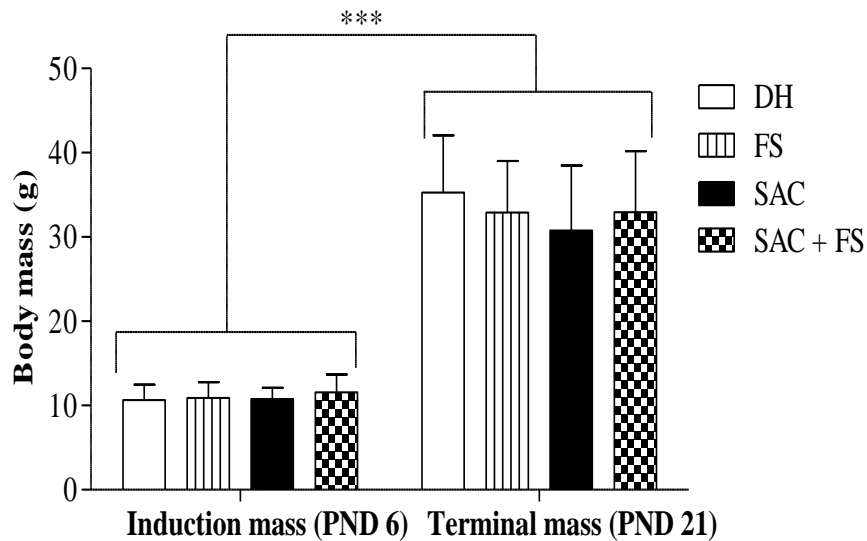


Figure 3.2: Effect of S-allyl-cysteine on terminal body masses of the female rat pups fed a high-fructose diet.

*** $p < 0.0001$. Induction body masses of the rats were similar across treatment regimens. Across treatment regimens, the rat pups significantly grew ($p < 0.0001$). However terminal body masses of the rats were similar across treatment regimens. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v); PND = postnatal day. Data presented as mean \pm SD; $n = 8$ per treatment.

Although the female rat pups across treatment regimens grew ($p < 0.0001$) significantly over the experimental period compared to their induction masses (Figure 3.2), the induction and terminal body masses of the female rat pups across treatment regimens were similar (Figure 3.2).

3.2.1.2 *Linear growth*

Table 3.1 below shows tibia masses, lengths and densities (Seedor indices) of male rat pups at the end of the study.

Table 3.1: Effects of S-allyl-cysteine on tibia lengths, masses and Seedor indices of suckling male rat pups fed a high-fructose diet

Parameter	DH	FS	SAC	SAC + FS	Significance level
Tibia length (mm)	18.60 ± 1.07	19.02 ± 1.61	19.10 ± 0.17	18.76 ± 1.58	ns
Tibia mass (mg)	32.50 ± 4.14	33.00 ± 2.88	33.00 ± 3.30	31.33 ± 2.50	ns
Tibia Seedor index (mg/mm)	1.75 ± 0.23	1.75 ± 0.22	1.73 ± 0.18	1.68 ± 0.21	ns

ns = not significant, $p > 0.05$. Tibiae lengths, masses and densities (Seedor indices) were similar for rats across the treatment regimens.

DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v). Data presented as mean ± SD; n = 8 per treatment regimen.

Tibiae masses, lengths, and densities (Seedor indices) of male pups were similar across the treatment regimens (Table 3.1).

Table 3.2 below shows tibia masses, lengths and densities (Seedor indices) of female rat pups at the end of the study.

Table 3.2: Effects of S-allyl-cysteine on tibia lengths, masses and Seedor indices of suckling female rat pups fed a high-fructose diet

Parameter	DH	FS	SAC	SAC + FS	Significance level
Tibia length (mm)	18.72 ± 1.27	18.30 ± 1.34	17.89 ± 1.53	17.84 ± 1.40	ns
Tibia mass (mg)	31.50 ± 12.38	33.13 ± 3.60	33.13 ± 3.36	31.63 ± 3.58	ns
Tibia Seedor index (mg/mm)	1.70 ± 0.21	1.81 ± 0.20	1.86 ± 0.27	1.78 ± 0.22	ns

ns = not significant, $p > 0.05$. Tibiae lengths, masses and densities (Seedor indices) were similar for rats across the treatment regimens.

DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v). Data presented as mean ± SD; n = 8 per treatment.

Tibiae masses, lengths, and densities (Seedor indices) of female pups were similar across treatment regimens (Table 3.2).

The male rat pups' radiographs of tibiae are shown in Figure 3.3 below.

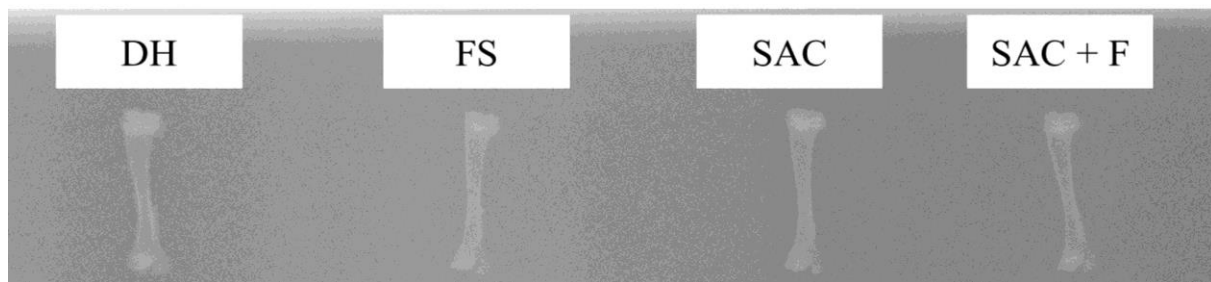


Figure 3.3: Radiograph images tibiae of male rat pups.

DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v).

There were no grossly subjectively observable differences in the radiographical densities of the tibiae from male rat pups across treatment regimens (Figure 3.3).

The female rat pups' radiographs of tibiae are shown in Figure 3.4 below.

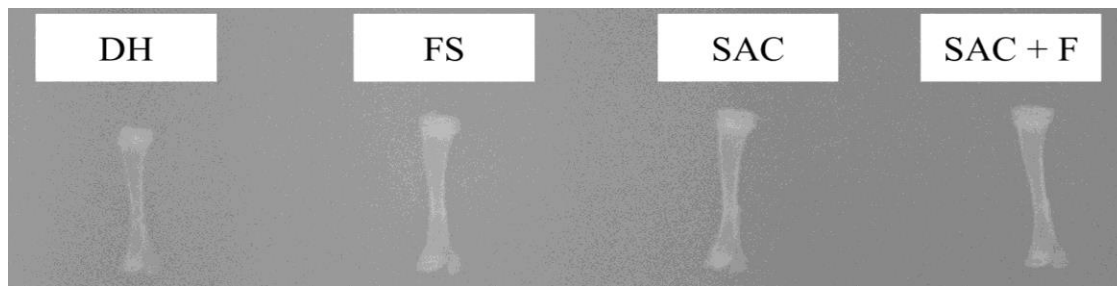


Figure 3.4: Radiograph images tibiae of female rat pups.

DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v).

There were no grossly subjectively observable differences in the radiographical densities of the tibiae from female rat pups across treatment regimens (Figure 3.4).

3.2.2 Blood parameters and hepatic metabolites

3.2.2.1 Non-fasted blood parameters

The blood glucose, triglyceride and cholesterol concentrations and plasma insulin concentration and HOMA-IR indices of male rat pups are shown in Table 3.3 below.

Table 3.3: Effect of S-allyl-cysteine on glucose, triglyceride, cholesterol and insulin concentrations, and HOMA-IR indices of non-fasted suckling male rat pups fed a high-fructose diet

Parameter	DH	FS	SAC	SAC + FS	Significance level
Glucose (mmol/L)	6.18 ± 0.51 ^a	6.47 ± 0.60 ^a	6.65 ± 0.36 ^a	6.15 ± 0.40 ^a	ns
Triglycerides (mmol/L)	3.94 ± 1.57 ^a	4.89 ± 1.31 ^a	3.82 ± 1.47 ^a	4.04 ± 1.21 ^a	ns
Cholesterol (mmol/L)	5.26 ± 0.78 ^a	4.70 ± 0.59 ^a	4.45 ± 0.41 ^a	4.70 ± 0.68 ^a	ns
Insulin (ng/mL)	24.78 ± 3.25 ^a	35.40 ± 9.87 ^{ab}	46.68 ± 11.13 ^b	34.68 ± 7.40 ^{ab}	***
HOMA-IR	7.43 ± 0.92 ^a	10.44 ± 3.06 ^{ab}	13.70 ± 2.94 ^b	9.47 ± 2.01 ^a	**

ns = not significant, $p > 0.05$. *** $p < 0.001$, ** $p < 0.01$. ^{ab}Within row means with different superscripts are significantly different at $p \leq 0.05$. Non-fasted blood glucose, triglyceride and cholesterol concentrations were similar in the rat pups across treatment regimens. Rat pups administered SAC alone had significantly higher ($p = 0.0006$) plasma insulin concentration compared to rats that were administered with DH. HOMA-IR was significantly higher ($p = 0.0009$ and $p = 0.0157$ respectively) in rats that were administered SAC alone compared to rats that were administered DH or SAC + FS. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v); HOMA-IR = Homeostatic model of insulin resistance. Data presented as mean ± SD; n = 8 per treatment.

There were no differences in the concentrations of the non-fasted blood glucose, triglyceride, and cholesterol of male pups across treatment regimens (Table 3.3). The administration of SAC significantly increased ($p = 0.0006$) male rat pups' plasma insulin concentration compared to male pups administered DH (control) (Table 3.3). Compared to male rat pups administered DH or SAC + FS, male rat pups administered SAC had significantly higher ($p = 0.0009$ and $p = 0.0157$, respectively) HOMA-IR (Table 3.3).

The blood glucose, triglyceride and cholesterol concentrations, plasma insulin concentration and HOMA-IR indices of female rat pups are shown in Table 3.4 below.

Table 3.4: Effect of S-allyl-cysteine on glucose, triglyceride, cholesterol and insulin concentrations, and HOMA-IR indices of non-fasted suckling female rat pups fed a high-fructose diet

Parameter	DH	FS	SAC	SAC + FS	Significance level
Glucose (mmol/L)	6.17 ± 0.43 ^a	6.00 ± 0.31 ^a	5.92 ± 0.75 ^a	6.22 ± 0.59 ^a	ns
Triglycerides (mmol/L)	4.36 ± 1.34 ^a	4.16 ± 0.82 ^a	3.63 ± 0.76 ^a	4.08 ± 1.18 ^a	ns
Cholesterol (mmol/L)	5.09 ± 0.68 ^a	4.95 ± 0.63 ^a	5.27 ± 0.75 ^a	4.88 ± 0.80 ^a	ns
Insulin (ng/mL)	37.90 ± 0.87 ^a	25.50 ± 4.14 ^b	44.96 ± 7.40 ^a	38.76 ± 6.34 ^a	**
HOMA-IR	10.60 ± 0.97 ^a	6.80 ± 1.43 ^b	11.99 ± 2.03 ^a	11.13 ± 1.94 ^a	**

ns = not significant, $p > 0.05$. ** $p < 0.01$. ^{ab}Within row means with different superscripts are significantly different at $p \leq 0.05$. Non-fasted blood glucose, triglyceride and cholesterol concentrations were similar in the rat pups across treatment regimens. Rats administered FS had significantly ($p < 0.01$) lower insulin and HOMA-IR compared to all other treatment regimens. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v); HOMA-IR = Homeostatic model of insulin resistance. Data presented as mean ± SD; n = 8 per treatment.

There were no significant differences in the concentration of the non-fasted blood glucose, triglyceride, and cholesterol concentration of male pups across treatment regimens (Table 3.4). The female rat pups to which a 20% FS was orally administered had significantly lower ($p < 0.01$) plasma insulin concentration and significantly lower ($p < 0.01$) HOMA-IR indices compared to their counterparts that were orally administered with DH, SAC or SAC + FS (Table 3.4).

3.2.2.2 Liver glycogen content

Liver glycogen (as glucose equivalent in liver homogenate) of male rat pups is shown in Figure 3.5 below.

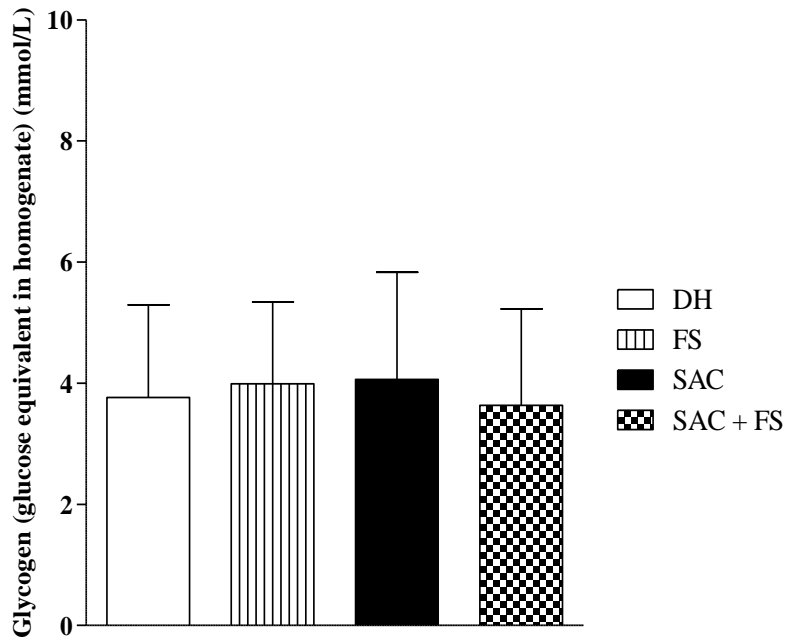


Figure 3.5: Effect of S-allyl-cysteine on the hepatic glycogen content of non-fasted suckling male rats fed high-fructose diet.

Liver glycogen (represented as glucose equivalents in the liver homogenate) content was similar in the rats across treatment regimens. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v). Data presented as mean \pm SD; n = 8 per treatment.

There were no significant differences in the liver glycogen content of male rat pups across treatment regimens (Figure 3.5).

Liver glycogen (as glucose equivalent in liver homogenate) of female rat pups is shown in Figure 3.6 below.

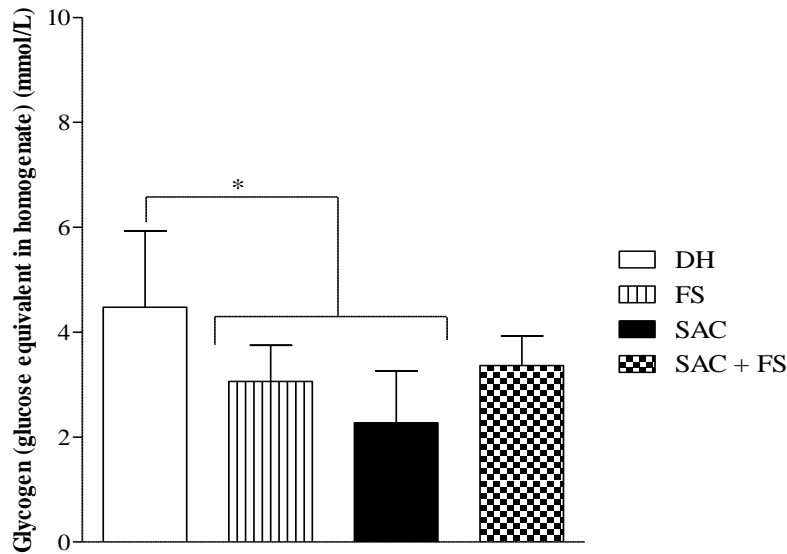


Figure 3.6: Effect of S-allyl-cysteine on the hepatic glycogen content of non-fasted suckling female rats fed high-fructose diet.

* $p < 0.05$. Rats orally administered FS only or SAC only had significantly lower ($p = 0.0177$ and $p = 0.0051$, respectively) liver glycogen (represented as glucose equivalents in the liver homogenate) compared to rats administered DH. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v). Data presented as mean \pm SD; $n = 7-8$ per treatment.

Unlike in male rat pups where the oral administration of FS and or SAC or the combination of FS and SAC had no effect on the liver glycogen content in female rat pups, the oral administration FS alone or SAC alone significantly reduced ($p = 0.0177$ and $p = 0.0051$, respectively) the liver glycogen content compared to female rat pups administered DH (Figure 3.6).

3.2.2.3 Liver lipid content

Figure 3.7 below shows the total hepatic lipid content of male rat pups.

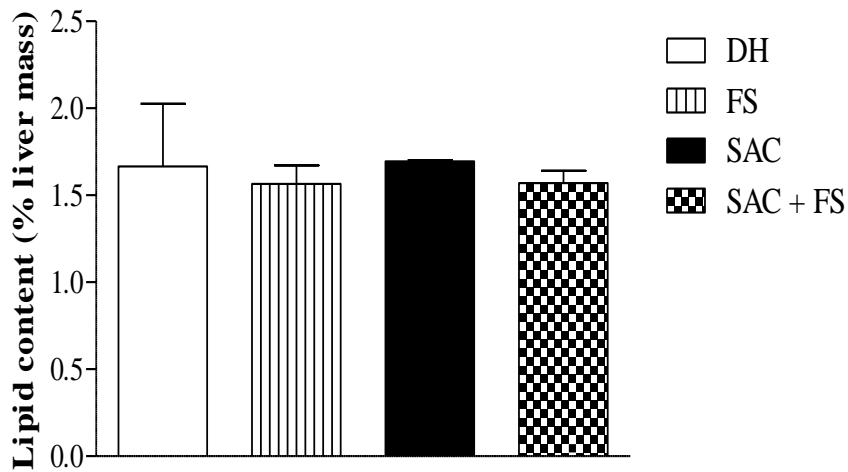


Figure 3.7: Effect of S-allyl-cysteine on the hepatic lipid content of non-fasted suckling male rats fed a high-fructose diet.

Liver lipid content was similar in all rats across treatment regimens. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v). Data presented as mean \pm SD; n = 8 per treatment.

The treatment regimens had no effect on male rat pups' liver lipid content (Figure 3.7).

Figure 3.8 below shows the total hepatic lipid content from female rat pups.

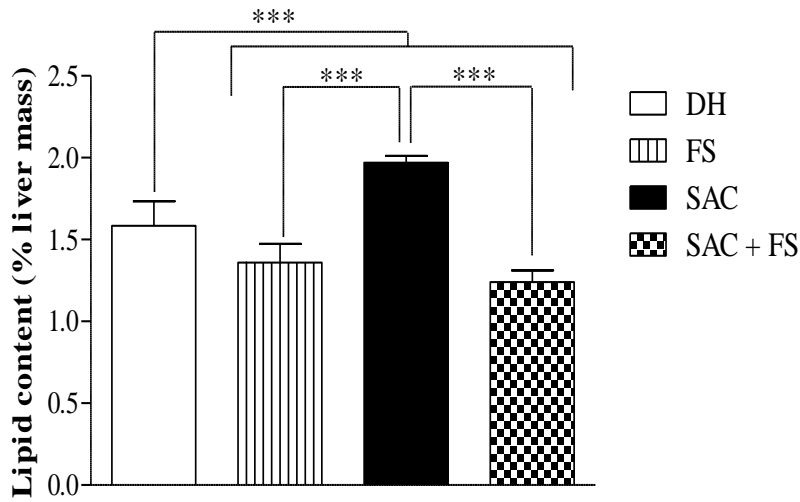


Figure 3.8: Effect of S-allyl-cysteine on the hepatic lipid content of non-fasted suckling female rats fed a high-fructose diet.

Rats administered FS or SAC + FS had significantly lesser ($p < 0.0001$) liver lipid content compared to rats administered DH or SAC. Liver lipid was significantly greater ($p < 0.0001$) in rats administered SAC compared to their counterparts administered DH. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v). Data presented as mean \pm SD; n = 8 per treatment.

The oral administration of 20% FS alone or SAC + FS in female rat pups resulted in significantly lesser ($p < 0.0001$) liver lipid content compared to the liver lipid content from female rat pups administered DH or SAC (Figure 3.8). In female rat pups, the administration of SAC caused a significant increase ($p < 0.0001$) liver lipid than in female rat pups administered DH (Figure 3.8).

3.2.3 Visceral organs morphometry

3.2.3.1 Liver, small and large intestine morphometry

Table 3.5 below shows the male rat pups' visceral organ absolute and relative (to tibia length) masses and lengths (small intestine and large intestine).

Table 3.5: Effect of S-allyl-cysteine on visceral organ masses and lengths in suckling male rats fed a high-fructose diet

Organ	DH	FS	SAC	SAC + FS	Significance level
Liver (g)	1.11 ± 0.30 ^a	1.35 ± 0.45 ^a	1.47 ± 0.25 ^a	1.39 ± 0.45 ^a	ns
Liver TLr (g/mm)	0.059 ± 0.012 ^a	0.070 ± 0.018 ^{ab}	0.081 ± 0.006 ^b	0.073 ± 0.019 ^{ab}	**
SI (mm)	496.30 ± 110.6 ^a	571.30 ± 73.00 ^a	567.20 ± 38.41 ^a	569.40 ± 62.47 ^a	ns
SI (g)	1.05 ± 0.27 ^a	1.32 ± 0.38 ^a	1.32 ± 0.27 ^a	1.32 ± 0.39 ^a	ns
SI TLr (g/mm)	0.056 ± 0.011 ^a	0.068 ± 0.014 ^a	0.072 ± 0.011 ^a	0.069 ± 0.016 ^a	ns
LI (mm)	83.75 ± 9.16 ^a	90.63 ± 12.08 ^a	91.67 ± 7.90 ^a	87.56 ± 15.69 ^a	ns
LI (g)	0.18 ± 0.04 ^a	0.21 ± 0.06 ^a	0.23 ± 0.04 ^a	0.21 ± 0.06 ^a	ns
LI TLr (g/mm)	0.009 ± 0.002 ^a	0.011 ± 0.002 ^a	0.012 ± 0.001 ^a	0.011 ± 0.002 ^a	ns

ns = not significant, $p > 0.05$. ** $p < 0.01$. ^{ab}Within row means with different superscripts are significantly different at $p \leq 0.05$. Rats administered SAC had significantly ($p = 0.0007$) heavier relative (tibia length) liver masses compared to the mass of the livers of rats administered DH. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v); LI = Large intestine; SI = small intestine; TLr = relative to tibia length. Data presented as mean ± SD; n = 8 per treatment.

The oral administration of SAC significantly increased ($p = 0.0008$) the male rat pups' relative liver masses (relative to tibia length) compared to the relative liver masses from male rat pups to which DH was orally administered (Table 3.5). Treatment regimens had no effect on male rat pups' absolute visceral organ (liver, small intestine and large intestine) masses and lengths (small intestine and large intestine) (Table 3.5).

Table 3.6 below shows the female rat pups' visceral organ (liver, small intestine and large intestine) absolute and relative masses (relative to tibia length) and lengths (small intestine and large intestine).

Table 3.6: Effect of S-allyl-cysteine on visceral organ masses and lengths in suckling female rats fed a high fructose diet

Organ	DH	FS	SAC	SAC + FS	Significance level
Liver (g)	1.20 ± 0.35	1.15 ± 0.36	1.04 ± 0.38	1.16 ± 0.37	ns
Liver TLr (g/mm)	0.063 ± 0.015	0.062 ± 0.015	0.058 ± 0.016	0.064 ± 0.017	ns
SI (mm)	535.00 ± 59.34	537.50 ± 66.49	514.40 ± 49.38	540.60 ± 71.29	ns
SI (g)	1.14 ± 0.31	1.16 ± 0.38	0.97 ± 0.30	1.12 ± 0.32	ns
SI TLr (g/mm)	0.062 ± 0.013	0.062 ± 0.017	0.054 ± 0.013	0.062 ± 0.014	ns
LI (mm)	86.25 ± 7.90	85.63 ± 9.04	85.63 ± 11.48	78.75 ± 8.34	ns
LI (g)	0.19 ± 0.04	0.18 ± 0.06	0.18 ± 0.05	0.18 ± 0.06	ns
LI TLr (g/mm)	0.009 ± 0.002	0.011 ± 0.003	0.013 ± 0.001	0.01 ± 0.002	ns

ns = not significant, $p > 0.05$. Visceral organ masses and lengths were similar in the rat pups across treatment regimens. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v); LI = Large intestine; SI = small intestine; TLr = relative to tibia length. Data presented as mean ± SD; n = 8 per treatment.

In female rat pups, treatment regimens had no effect on absolute and relative (relative to tibia length) visceral organ (liver, small intestine and large intestine) masses and lengths (small intestine and large intestine) (Table 3.6).

3.2.3.2 *Liver histo-morphometry*

Table 3.7 below shows hepatocyte cell size and numbers of hepatocytes in representative linear fields (100 μm) of male rat pups.

Table 3.7: Effects of S-allyl-cysteine on hepatocyte size and density of suckling male rat pups fed a high-fructose diet

Parameter	DH	FS	SAC	SAC + FS	Significance level
Hepatocyte size (μm)	7.83 ± 1.15	5.50 ± 1.32	7.00 ± 2.18	7.00 ± 0.50	ns
Hepatocyte density (cells per $100 \mu\text{m}$)	16.83 ± 0.76	16.50 ± 0.50	16.17 ± 1.26	16.67 ± 1.53	ns

ns = not significant, $p > 0.05$. The number of intact hepatocytes in a linear field ($100 \mu\text{m}$) and hepatocyte size were similar in the rat pups across treatment regimens. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v). Data presented as mean \pm SD; $n = 3$ per treatment.

Hepatocyte cell size and numbers of hepatocytes in a 100 μ m linear field were similar across treatment regimens in male pups (Table 3.7).

Table 3.8 below shows hepatocyte cell size and numbers of hepatocytes in representative linear fields (100 μm) of female rat pups.

Table 3.8: Effects of S-allyl-cysteine on hepatocyte size and density of suckling female rat pups fed a high-fructose diet

Parameter	DH	FS	SAC	SAC + FS	Significance level
Hepatocyte size (μm)	4.83 ± 1.15	5.16 ± 1.60	6.66 ± 1.60	6.33 ± 0.58	Ns
Hepatocyte density (cells per 100 μm)	17.00 ± 2.78	18.83 ± 1.26	20.17 ± 1.53	17.00 ± 2.00	Ns

ns = not significant, $p > 0.05$. The number of intact hepatocytes in a linear field (100 μm) and hepatocyte size were similar in the rat pups across treatment regimens. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v). Data presented as mean \pm SD; n = 3 per treatment.

Hepatocyte size and numbers of hepatocytes in a 100 μ m linear field were similar across treatment regimens in female pups (Table 3.8).

Non-alcoholic fatty liver disease activity score (NAS) of male rats after being administered their respective treatment regimens are shown in Table 3.9 below.

Table 3.9: Effects of S-allyl-cysteine on non-alcoholic fatty liver disease activity score (NAS) of suckling male rat pups fed a high-fructose diet

Parameter	DH	FS	SAC	SAC + FS	Significance level
Steatosis score	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)	ns
Ballooning score	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)	ns
Lobular inflammation score	2 (2, 2)	2 (2, 2)	1 (1, 2)	2 (2, 3)	ns
NAS	2 (2, 2)	2 (2, 2)	1 (1, 2)	2 (2, 3)	ns

ns = not significant. The non-alcoholic fatty liver disease activity scores (NAS) of male rats were similar across treatment regimens. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v); NAS = non-alcoholic fatty liver disease activity score. Total NAS is the sum of values recorded for each category. Total NAS score interpretation: <2 = not steatohepatitis; 3–4 = uncertain; >5 = probable or definite steatohepatitis. Data presented as median and range (min, max); n = 3 per treatment.

The treatment regimens had no effect on NAS of male rat pups (Table 3.9).

Non-alcoholic fatty liver disease activity score (NAS) of female rat pups after being administered their respective treatment regimens are shown in Table 3.10 below.

Table 3.10: Effects of S-allyl-cysteine on non-alcoholic fatty liver disease activity score (NAS) of suckling female rat pups fed a high-fructose diet

Parameter	DH	FS	SAC	SAC + FS	Significance level
Steatosis score	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)	ns
Ballooning score	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)	ns
Lobular inflammation score	1 (1, 3)	1 (1, 2)	2 (2, 2)	2 (2, 2)	ns
NAS	1 (1, 3)	1 (1, 2)	2 (2, 2)	2 (2, 2)	ns

ns = not significant. The non-alcoholic fatty liver disease activity scores (NAS) of female rats were similar across treatment regimens. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v); NAS = non-alcoholic fatty liver disease activity score. Total NAS is the sum of values recorded for each category. Total NAS score interpretation: <2 = not steatohepatitis; 3–4 = uncertain; >5 = probable or definite steatohepatitis. Data presented as median and range (min, max); n = 3 per treatment.

The treatment regimens had no effect on NAS of female rat pups (Table 3.10).

Representative liver histology photo sections (H and E staining, 400 X magnification) of male rat pups from the different treatment groups are shown in Figure 3.9 below.

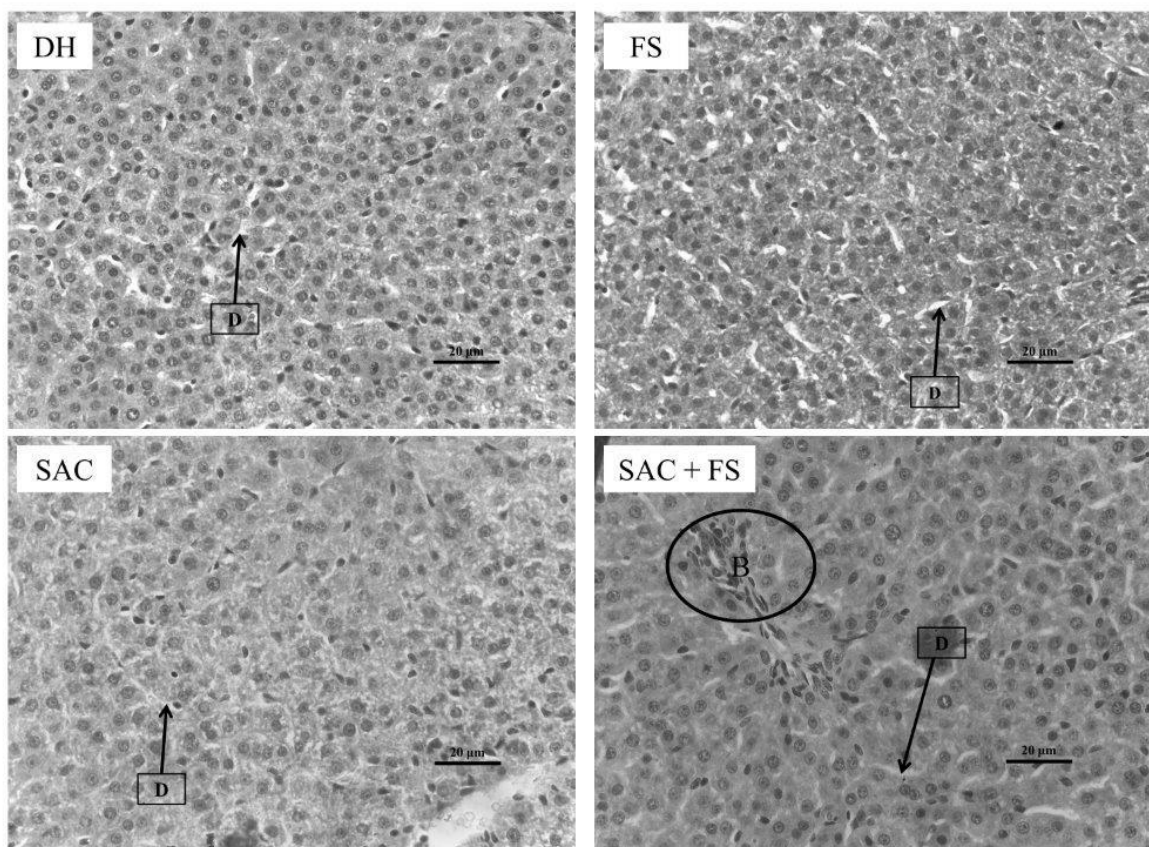


Figure 3.9: Photos of sections showing the liver histology (H and E staining, 400 X magnification) of suckling male rats following the treatment regimens.

Circle B shows foci of lobular inflammation and arrows D point to sinusoids. No histological differences were noted in the liver samples from rats across treatment regimens. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v).

Liver histology of the male rat pups was similar across treatment regimens and no pathology was noted (Figure 3.9).

Representative liver histology photo sections (H and E staining, 400 X magnification) of female rat pups from the different treatment groups are shown in Figure 3.10 below.

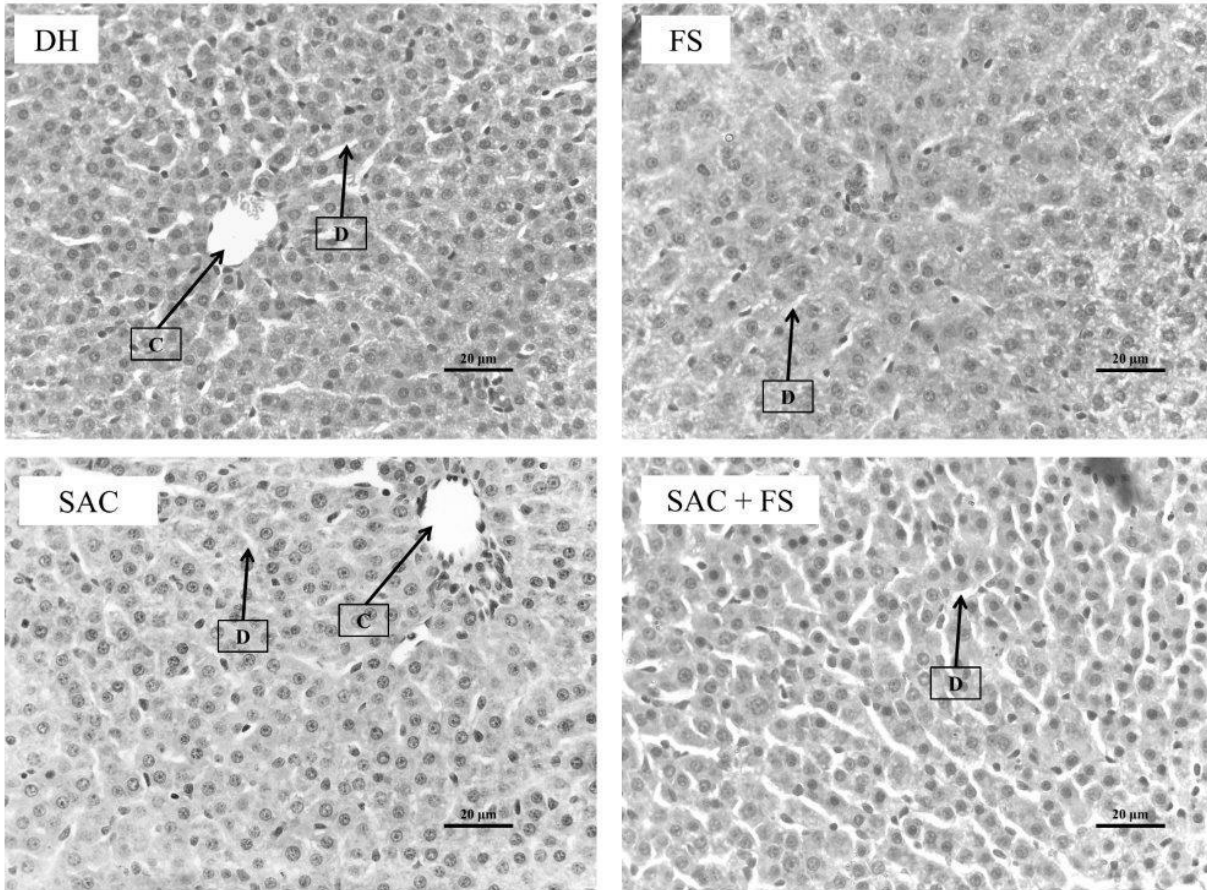


Figure 3.10: Photos of sections the liver histology (H and E staining, 400 X magnification) of suckling female rats following the treatment regimens.

Arrows C and D point to central veins and sinusoids respectively. No differences were noted in the liver histology across treatment regimens. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v).

Liver histology of the female rat pups was similar across treatment regimens and no pathology was noted (Figure 3.10).

3.2.4 Plasma surrogate markers of liver and kidney function

Table 3.11 below shows the male rat pups' plasma activity of alanine aminotransferase and alanine phosphatase and plasma concentrations of total bilirubin, globulin, albumin, blood urea nitrogen, and creatinine.

Table 3.11: The effect of S-allyl-cysteine on plasma surrogate biomarkers of health in suckling male rats fed a high-fructose diet

Parameter	DH	FS	SAC	SAC + FS	Significance level
ALT (U/L)	49.33 ± 8.33 ^a	62.17 ± 13.45 ^a	64.50 ± 12.53 ^a	66.00 ± 7.01 ^a	ns
ALP (U/L)	187.8 ± 34.71 ^a	209.50 ± 25.54 ^a	203.50 ± 22.45 ^a	211.3 ± 23.20 ^a	ns
Total bilirubin (mg/dL)	7.83 ± 3.65 ^a	5.33 ± 1.96 ^a	3.67 ± 1.03 ^a	5.83 ± 3.43 ^a	ns
Globulin (g/dL)	23.50 ± 1.22 ^a	23.83 ± 1.17 ^a	22.00 ± 3.16 ^a	22.67 ± 1.50 ^a	ns
Albumin (g/dL)	21.00 ± 1.25 ^a	22.67 ± 1.50 ^{ab}	25.00 ± 1.55 ^b	24.83 ± 1.33 ^b	***
Total protein (g/dL)	44.83 ± 2.23 ^a	46.83 ± 2.23 ^a	47.00 ± 2.09 ^a	47.50 ± 1.38 ^a	ns
Blood urea nitrogen (mg/dL)	6.25 ± 0.37 ^a	5.83 ± 0.49 ^a	6.43 ± 1.04 ^a	6.43 ± 1.13 ^a	ns
Creatinine (mg/dL)	15.00 ± 4.65 ^a	10.50 ± 3.67 ^a	11.67 ± 4.18 ^a	10.50 ± 3.67 ^a	ns

ns = not significant, ***p < 0.0001. ^{ab}Within row means with different superscripts are significantly different at p < 0.05. Rats administered SAC or SAC + FS had significantly (p < 0.0001) higher plasma albumin concentration compared to the plasma albumin concentration of rats administered DH. DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v); ALP = Alanine phosphatase ALT = Alanine aminotransferase. Data presented as mean ± SD; n = 8 per treatment.

The oral administration of SAC alone or SAC + FS significantly increased ($p < 0.0001$) plasma albumin concentration in male rat pups compared to the oral administration of DH (Table 3.11). Plasma activity of alanine aminotransferase and alanine phosphatase and plasma concentrations of total bilirubin, globulin, blood urea nitrogen, and creatinine were similar in male rat pups across treatment regimens (Table 3.11).

Table 3.12 below shows the female rat pups' plasma activity of alanine aminotransferase and alanine phosphatase and plasma concentrations of total bilirubin, globulin, albumin, blood urea nitrogen, and creatinine.

Table 3.12: The effect of S-allyl-cysteine on plasma surrogate biomarkers of health in suckling female rats fed a high-fructose diet

Parameter	DH	FS	SAC	SAC + FS	Significance level
ALT (U/L)	47.67 ± 11.45	50.67 ± 15.67	45.33 ± 10.97	60.33 ± 14.31	ns
ALP (U/L)	206.5 ± 40.08	185.70 ± 17.67	155.50 ± 47.04	178.0 ± 27.03	ns
Total bilirubin (mg/dL)	5.00 ± 2.53	6.50 ± 2.34	7.16 ± 2.71	6.66 ± 5.53	ns
Globulin (g/dL)	23.50 ± 1.51	24.17 ± 1.33	25.00 ± 2.45	23.00 ± 2.30	ns
Albumin (g/dL)	22.00 ± 2.00	22.17 ± 1.94	22.33 ± 4.45	21.67 ± 3.14	ns
Total protein (g/dL)	45.83 ± 2.48	46.17 ± 3.54	47.00 ± 2.76	44.67 ± 1.63	ns
Blood urea nitrogen (mg/dL)	6.80 ± 0.93	7.22 ± 1.80	6.08 ± 0.80	6.02 ± 0.88	ns
Creatinine (mg/dL)	10.50 ± 3.67	13.50 ± 4.93	12.00 ± 4.65	13.50 ± 4.93	ns

ns = not significant $p > 0.05$. All surrogate markers of liver and kidney function were similar in the rat pups across treatment regimens.

DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v); ALP = Alanine phosphatase ALT = Alanine aminotransferase. Data presented as mean ± SD; n = 8 per treatment.

The treatment regimens had no effect on plasma activity of alanine aminotransferase and alanine phosphatase and plasma total bilirubin, globulin, albumin, blood urea nitrogen, and creatinine concentration of female rat pups (Table 3.12).

3.3 Discussion

3.3.1 Growth performance

Although the terminal body masses of male or female rat pups across treatment regimens were similar, both male and female rat pups significantly grew compared to their induction masses (Figures 3.1 and 3.2). These findings suggest that the treatment regimens had no adverse effects on the growth performance of the male and female rat pups as measured by mass. It is important to note though that, as a measure of growth, body mass is not an accurate index as it is affected transiently by visceral/subcutaneous obesity, hydration status, and gut fill (Cameron, 2002) as well as the thickness of the integument and size of integumentary appendages (Vítek *et al.*, 2008). Long bones grow in response to growth hormone in a dose-dependent manner and hence long bone linear growth is a more accurate long-term measure of growth performance (Butler *et al.*, 1956) before fusion of the growth plates. The similarity in the tibial lengths, masses and Seedor indices from the rat pups (male and female) across treatment regimens further suggest that the treatment regimens had no adverse effect on the growth performance of rat pups.

3.3.2 Blood parameters and hepatic metabolites

In the current study, the oral administration of 10 ml/kg body mass per day of 20% fructose solution (FS) for 15 days to suckling male or female rat pups did not result in hyperglycaemia, hypertriglyceridemia, and hypercholesterolemia. This suggests that the administration of the 20% fructose solution during this neonatal growth phase did not induce metabolic derangements with regards to glucose and lipid metabolism. On the contrary studies using adult rat models, documented that the consumption of high-fructose diets for 2-12 weeks resulted in increased lipogenesis, atherogenic dyslipidaemia (visceral obesity, hepatic hyperlipidaemia, hypercholesterolemia, and hypertriglyceridemia), hyperglycaemia and insulin resistance (Huynh *et al.*, 2008; Lindqvist *et al.*, 2008; Thorburn *et al.*, 1989). *Ghezzi et al. (2012) and de Moura et al. (2009) contend that since at a young age Wistar rats are more resistant to the development of diet-induced derangements as they are less prone to being obese than older animals. Obesity is a major risk factor for diet-induced metabolic derangements (Mager et al., 2015).* Several possible explanatory mechanisms have been proposed. Included amongst the possible mechanisms are that at a young age, GLUT 4

transporters that are responsible for the uptake of glucose are less likely to be dysfunctional (Ghezzi *et al.*, 2012; Machado *et al.*, 1994) resulting in an efficient uptake of glucose. In addition, in young rats, intestinal brush border fructose transporters (GLUT 5) are decreased and immature: they start to increase and mature post-weaning (Jiang *et al.*, 2001). Thus the observed lack of derangements in blood glucose concentration and lipid profile following the oral administration of a 20% fructose solution during the neonatal growth phase could be ascribed to functional capacities of the GLUT 4 and GLUT 5 transporters during the period of intervention. Whilst it is notable that the introduction of fructose at pre-weaning (suckling) has been shown to promote the precocious development of GLUT 5 transporters (David *et al.*, 1995), it is not possible to draw definitive conclusions from the findings of the current study regarding the ability of the orally administered 20% fructose solution during neonatal growth phase on the induction of precocious maturation of GLUT 5 transporters. Future studies would require the measurement of the GLUT 5 transporters in under similar study settings.

The current study also reports reduced plasma insulin concentrations in female rat pups to which 20% fructose solution was orally administered for 15 days (Table 3.4). This finding suggests that oral administration of a 20% fructose solution to suckling female Wistar rat pups may have exerted anti-insulinotropic effects in female rat pups but not in their male counterparts. According to our knowledge, no study has reported on the possible inhibitory effects of a high-fructose diet (in this case a 20% fructose solution) to insulin secretion by either young and or adult rats. The difference in the response to neonatal orally administered 20% fructose solution by the male and female rats pup indicates the existence of sex- and age-related variations to a fructose insult. The consumption of high-fructose diets has been shown to decrease postprandial satiety, leptin and insulin response in normal-weight human adults (Teff *et al.*, 2004). These decreased responses have been associated with a reduction in circulating insulin concentrations in normal-weight human adults (Teff *et al.*, 2004; Kong *et al.*, 1999). Insulin stimulates satiety (Melanson *et al.*, 2008). Alzamendi *et al.* (2010) hypothesised that orally administered fructose in early-life (gestation and lactation) dysregulates hypothalamic control of satiety. Thus we hypothesise that the oral administration of a 10 ml/kg body mass per day of 20% fructose solution for 15 days during the neonatal growth phase (suckling) may have altered satiety control through the dysregulation of hypothalamic control of satiety, leptin and insulin response and consequently caused decreased plasma insulin concentration in the female rat pups. Research

has demonstrated that young female rats are more sensitive than young male rats to the adverse metabolic programming outcomes of maternal high-fructose diets (Vicker *et al.*, 2011; Ojeda *et al.*, 2016). Nonetheless, the mechanisms associated with our findings on female rat pups' sexually dimorphic insulin response to high-fructose diet require further interrogation. The oral administration of SAC + FS had no effect on plasma insulin concentration which suggests that SAC attenuated insulin-lowering effects of a 20% fructose solution seen in female rat pups. We speculate that the mechanisms by which SAC attenuated the insulin-lowering effects of a 20% fructose solution might have been associated with its reported insulinotropic effects (Saravanan *et al.*, 2009; Augusti and Sheela, 1996).

Whilst Saravanan *et al.* (2009) reported that SAC attenuates hyperglycaemia, hypercholesterolemia and hypertriglyceridemia in STZ-diabetic adult rats, Liu and Yeh (2001) noted *in vitro* that SAC inhibited fatty acid and triglyceride synthesis in cultured rat hepatocyte cells. It is proposed that SAC exerts its antidiabetic and antiobesity effects by binding to and blocking the insulin-inactivating sites in the liver and thus increasing the availability of free insulin (Augusti and Sheela, 1996). In the current study, the oral administration of SAC alone had no effect on female pups' blood glucose, cholesterol and triglyceride concentration.

It should be noted that although the plasma insulin concentrations of suckling female rat pups to which SAC and DH (control) was orally administered were not significantly different, the female rat pups to which SAC was orally administered showed a trend of a higher insulin concentration compared to their counterparts on DH. The oral administration of SAC alone increased plasma insulin concentration and HOMA-IR in male rat pups but had no effect on their blood glucose, cholesterol, and triglyceride concentration (Table 3.3). It could be inferred from the results that orally administered SAC's effects on insulin secretion are sexually dimorphic. These findings seem to confirm the antidiabetic activity of SAC and hence its potential use as an antidiabetic agent. These findings are in agreement with those by Saravanan *et al.* (2009) who noted that SAC attenuated hypoinsulinemia in STZ-diabetic male adult rats. S-allyl-cysteine is reported to stimulate insulin secretion by pancreatic β -cells and prevent insulin inactivation (Saravanan *et al.*, 2009).

The sexually dimorphic response to SAC observed in suckling rat pups may be related to the variation in the timing of development of physiological systems. In male rats, an upsurge in testosterone in late gestation and neonatal periods has been shown to accelerate development

of various brain structures including the hypothalamic system (Sakuma, 2009) and consequently resulting in the earlier development of the endocrine systems (Viveros *et al.*, 2009). In addition, greater incretins concentrations have been reported in male rat pups than in female rat pups (Ojeda *et al.*, 2016). Therefore the sexually dimorphic responses to orally administered SAC and or the 20% fructose solution (simulating a high-fructose diet) observed in the suckling rats could be due to the sex-related variation in the development of the endocrine system.

In the present study, the oral administration of 20% fructose solution alone or SAC + FS resulted in a significant reduction in the liver lipid content of female rat pups (Figure 3.8). Additionally, the liver glycogen content was reduced in female rat pups to which a 20% fructose solution alone was orally administered in the present study (Figure 3.6). These findings suggest that the orally administered 20% fructose solution may have altered synthesis and storage of liver metabolites. The findings from the present study are in disagreement with Araujo *et al.* (2015) and Yadav *et al.* (2007), who reported that the consumption of a high-fructose diet (10% fructose w/v) resulted in increased hepatic lipid and glycogen content in adult male rats. However, this disagreement in findings could be attributed to the differences in the age and sex of the rats that were used in the current study (21-days old) and that by Araujo *et al.* (2015) and Yadav *et al.* (2007) who used adult rats.

Insulin, an anabolic hormone, stimulates tissues' uptake of glucose and promotes lipogenesis in the liver and adipose tissue (Samuel and Shulman, 2012). Thus the reduced hepatic lipid and glycogen content observed in female rat pups to which a 20% fructose solution was orally administered could be attributed to the reduced insulin observed in them (Table 3.4). The treatment regimens administered in the current study had no effect on male rat pups' liver glycogen and lipid content further suggesting that female rat pups could be more susceptible to the metabolic effects of high-fructose diet during suckling when compared to their male counterparts. Our findings are similar to those reported by Ojeda *et al.* (2016) who observed that in early-life female rat pups were more sensitive than males to adverse effects of a maternal high-fructose diet.

Interestingly, the present study also reports that in female rat pups the administration of SAC alone for 15 days increased liver lipid content and decreased liver glycogen content (Figures 3.6 and 3.8). We hypothesise that the changes in liver glycogen and lipid content in female rat pups might have been triggered by the SAC's speculated insulinotropic effects (Iliya *et*

al., 2016a; Saravanan *et al.*, 2009). Insulin, a major metabolic regulatory hormone, stimulates glycogenesis and lipogenesis and inhibits lipolysis in tissues (Samuel and Shulman, 2012). Koopmans *et al.* (1999) reported that in adult Sprague-Dawley rats, prolonged hyperinsulinemia stimulated *de novo* lipogenesis, inhibited glycogenesis and triggered insulin resistance. Insulin-activated glycogen synthesis is inhibited by excess lipid accumulation in the liver (Samuel and Shulman, 2012), hence this may explain the decreased glycogen noted in suckling female rats that were orally administered SAC in the current study.

The potential beneficial effects of SAC (insulinotropic effect) could possibly result in adverse metabolic effects such as increased hepatic lipid accumulation and consequently NAFLD in growing females. An increase in liver lipid content could potentially cause NAFLD and subsequently increase the risk of developing metabolic derangements such as MetS, DM II, and cardiovascular complications. *In vitro* work by Lui and Yeh (2001) demonstrated that SAC inhibits cholesterol and free fatty acid synthesis in cultured liver cells. In non-alcoholic fatty liver diseased 29-day old Otsuka Long-Evans Tokushima male rats, the consumption of SAC (0.45% w/v in feed) upregulated hepatic proliferator-activated receptors alpha and gamma resulting in mitigated hypertriglyceridemia, hypercholesterolemia and NAFLD (Takemura *et al.*, 2013). Our results are in disagreement with those of Takemura *et al.* (2013) and Lui and Yeh, (2001). The difference between our results and those of Takemura *et al.* (2013) could be attributed to differences in the physiological effects of SAC in neonatal and adult rats, experimental model [preventative model used by the current study versus the treatment model used by Takemura *et al.* (2013)] and doses used [150 mg/kg body mass per day of SAC used in the current study versus 0.45% SAC in feed used by Takemura *et al.* (2013)]. The demonstrated lipogenic effect of SAC in female rats' livers during neonatal life in the present study requires further scrutiny in order not only to elucidate fully the underlying physiological mechanisms but to explore the possible long-term implications an issue which is partially investigated in the study described in chapter four of this thesis.

3.3.3 Visceral organs

Orally administered substances can alter growth and development of visceral organs such as the liver and GIT (Metges, 2011). While the GIT is vital for food digestion and nutrient absorption, the liver is regarded as the metabolic powerhouse (Metges, 2011). The current

study shows that in male rat pups, the oral neonatal administration of SAC resulted in significantly heavier relative (to tibia length) liver masses compared to rat pups to which distilled water (control) was administered (Table 3.5). Despite the observed heavier liver masses in male rat pups (relative to tibia length) as a result of neonatal oral administration of SAC; this administration of SAC did not result in an increase in hepatocyte size and number of intact hepatocytes in a linear field in both male and female rat pups. Whilst this finding suggests that SAC may have stimulated liver growth in male rat pups but not in female rat pups hence the former's heavier livers (relative to tibia length). However, it is unlikely since there were no differences in the absolute masses of the rat pups' livers. The observed differences could possibly be due to the differences in bone lengths which although not statistically significantly different showed a trend to being slightly smaller in the DH (control) groups.

The liver is a major site of metabolism and hence is prone to toxic insults which can be assessed histologically. The present study reports no differences in hepatic ballooning, inflammation and steatosis scores and NAS and no observable pathological differences in the micro-morphometry of the liver samples from the male and female rat pups across treatment regimens. These findings suggest that none of the treatment regimens had adverse effects on the hepatic histological morphometry of male and female rat pups. Remarkably, although neonatal orally administered SAC induced increased total liver lipid content in female rat pups, this did not translate to adverse liver histomorphometric and pathological effects when scored using established criteria.

Studies have shown that administration to neonates of phytochemicals such as phytohaemagglutinin, polyamines and allicin induces precocious maturation of the GIT (Tatara *et al.*, 2008; Linderoth *et al.*, 2006; Linderoth *et al.*, 2005; Löser *et al.*, 1999). The similarities in absolute and relative (to tibia length) masses and lengths of the small intestine and large intestine of male and female rat pups across treatment regimens suggests that the neonatal oral administration of DH, SAC, FS and or SAC + FS had no adverse effects on the small and large intestines macro-morphometry. Although both SAC and allicin are phytochemicals derived from garlic, unlike allicin the SAC did not impact on the gross morphometry of the GIT. Hence further supporting the biosafety of SAC compared to allicin (Amagase, 2006; Amagase *et al.*, 2001).

3.3.4 Surrogate markers of liver and kidney function

The use of ethnomedicinal-derived phytochemicals to manage systemic ailments has various potential adverse consequences such as cytotoxicity and organ damage (Nehar *et al.*, 2015). Thus when investigating the potential of ethnomedicinal-derived phytochemicals it is essential to also interrogate their probable detrimental health effects. The liver, regarded as the metabolic engine, has various functions including synthesis and storage of metabolites and hormones (Samuel and Shulman, 2012), detoxification of xenobiotic substances (Skrypnik *et al.*, 2016) and conjugation of bilirubin (Erlinger *et al.*, 2014). Through these functions, it is exposed to many chemicals and toxins presented to the body.

While increased plasma activity of ALT is indicative of intra-hepatic damage, depending on the isoform that is increased, plasma ALP activity is associated with post-hepatic liver damage, increased bone turnover and or inflammatory intestinal diseases (Lens *et al.*, 2014; Philip *et al.*, 2014; Schutte *et al.*, 2013). Bilirubin, a waste product formed from haemolysis, is solubilised in the liver and excreted in the faeces and urine. Increased plasma bilirubin concentrations are indicative of pre-hepatic, intra-hepatic and post-hepatic liver disease (Pratt and Kaplan, 2000). In mice, SAC protects against acetaminophen-induced hepatotoxicity by mitigating oxidative stress induced damage and attenuating C-reactive protein (CRP) and ALT activity (Hsu *et al.*, 2006). The neonatal oral administration 20% fructose solution, SAC and or SAC + FS had no effect on the surrogate markers of liver function (ALT, ALP, and total TBIL) in the male and female rat pups. These findings suggest that the neonatal orally administered of 20% fructose solution, SAC and or SAC + FS had no toxic adverse effects on the liver function of both the male and female rat pups.

Albumin and globulin, major plasma proteins synthesised by the liver, are responsible for numerous physiological functions (Azab *et al.*, 2013). Decreased albumin and globulin plasma concentration are associated with advanced liver damage (Sekhar *et al.*, 2014). Hyperalbuminemia commonly occurs in patients with severe dehydration (Rybakowski *et al.*, 2012; Shane *et al.*, 2010) and it may also be induced by high-protein intake (Mutlu *et al.*, 2006). In the current study male rat pups to which SAC and or SAC + FS was orally administered during the neonatal growth phase had increased plasma albumin concentration (Table 3.11). Although the current study did not directly evaluate the hydration status of the rat pups, there were no overt clinically signs of dehydration such as decreased skin elasticity and dry mucous membranes (Chisti *et al.*, 2013). Thus our findings of hyperalbuminemia

noted in suckling male rat pups that were orally administered SAC may be attributed to SAC probably altering albumin metabolism in male rat pups. Our findings are in agreement with Saravanan and Ponmurugan, (2012b), who reported that orally administered SAC (150 mg/kg) prevented hypoalbuminemia in STZ-diabetic adults Wistar rats by inhibiting protein catabolism and promoting albumin synthesis and secretion. It is unclear why this was noted in male rat pups only in the current study.

The kidneys are responsible for various important physiological functions such as producing hormones, reabsorbing glucose and minerals, excreting urea, creatinine and sodium and producing urine. Whereas urea is synthesised from ammonia generated either by oxidative breakdown of amino acids and or via transamination reaction in the liver (Wyss and Kaddurah-Daouk, 2000), creatinine is produced as a breakdown product of creatinine kinase in skeletal and cardiac muscle (Macedo *et al.*, 2010; Beddhu *et al.*, 2003). Urea and creatinine are filtered by the kidney from blood and are excreted in urine (Brisco *et al.*, 2013). As such they are used as surrogate markers of kidney function thus any impairment to renal function results in increased plasma urea and creatinine (Cavalcanti *et al.*, 2006). An assessment of the urea:creatinine ratio is used to classify the type of renal impairment, namely prerenal, intrarenal and postrenal (Brisco *et al.*, 2013). In the current study, the treatment regimens had no effect on the plasma creatinine and blood urea nitrogen of pups suggesting that they (treatment regimens) did not have any toxic or adverse effects on the kidneys of suckling male and female rat pups.

3.4 Conclusion

Orally administered S-allyl-cysteine exhibited no toxicity to the liver and kidneys of suckling Wistar rat pups. Neonatal (suckling) oral administration of a 20% fructose solution or SAC resulted in sexually dimorphic effects on the rat pups' plasma insulin concentration and hepatic glycogen and lipid storage. Specifically, the neonatal oral administration of a 20% FS to suckling female rat pups caused hypoinsulinemia which, (hypoinsulinemia) was attenuated by the neonatal oral administration of SAC. While in male rat pups the neonatal oral administration of SAC alone increased plasma insulin concentration, in female rat pups it increased hepatic lipid accretion. Although SAC exhibited insulinotropic potential in male and female rat pups which make it a probable antidiabetic agent, its use in young females

should be done with caution as it may increase the risk of developing NAFLD as it was observed to increase liver lipid content in female rat pups that were modelling human infants.

The next chapter (chapter 4) provides background literature and a justification and then states the aim, objectives and hypotheses of the second experiment which sought to interrogate the long-term effects and the potential of neonatal orally administered SAC to protect against a 'single-hit' and 'double-hit', high-fructose diet-induced metabolic derangements. The chapter also details the materials and methods and narrates the findings of the study and discusses the findings of the second experiment.

**CHAPTER 4: EFFECTS OF EARLY
ADMINISTRATION OF S-ALLYL-
CYSTEINE ON THE RESPONSE TO A
HIGH FRUCTOSE DIET**

4.0 Introduction

Children that are overweight or obese are most likely to grow up to become obese adults (Pandita *et al.*, 2016). This suggests that metabolic derangements in early-life may play a key role in the epidemic prevalence of obesity in adulthood. Globally, approximately 2.1 billion adults are overweight or obese (Smith and Smith, 2016). Obesity increases morbidity by raising the risk of developing cardio-metabolic complications such as NAFLD, MetS, DM II, coronary heart disease and ischaemic heart failure (Reinehr, 2016; Alisi and Nobili, 2014; Sookoian *et al.*, 2013; Cole *et al.*, 2000). The consumption of unhealthy diets, sedentary lifestyles, a reduction in sleep, urbanisation, stressful environment, medical conditions (for example hypothyroidism), genetic predisposition socio-economic status and neonatal exposure to suboptimal diets which results in epigenetic changes and metabolic programming contribute to the onset and maintenance of obesity and its consequent metabolic derangements (Franks and McCarthy, 2016; Mohamed *et al.*, 2014).

Investigations into the lifestyle, environmental, genetic and epigenetic factors that contribute to the obesity epidemic are essential in the effort to find interventions to prevent, manage, and treat obesity and its numerous consequential complications. Researchers have focused on investigating the role of lifestyle and genetic factors on the increased prevalence of obesity, NAFLD and MetS (Albracht-Schulte *et al.*, 2016; Reinehr, 2016). However the Developmental Origins of Health and Disease (DOHaD) hypothesis states that perinatal exposure to suboptimal nutritional and environmental stimuli can cause epigenetic changes that alter the functioning of various physiological systems and consequently increase the susceptibility or resistance of developing metabolic derangements in adulthood (Nomura and Yamanouchi, 2012; Vickers *et al.*, 2011; Chen *et al.*, 2010). Epigenetic changes result in the upregulation and or down-regulation of gene expression without altering the genome sequence (Laker *et al.*, 2013). The main mechanisms that cause epigenetic changes are methylation of the DNA and histone modification (Vickers *et al.*, 2011). The methylation of DNA entails the addition of methyl groups to the fifth carbon of the cytosine ring (Laker *et al.*, 2013) while histone modification results from the alteration of DNA packaging proteins through processes such as acetylation, methylation and or phosphorylation (Miao *et al.*, 2014). The changes in the DNA structure through methylation and or changes in the DNA packaging proteins have been and are linked to the single-hit and double-hit hypotheses of diet-induced metabolic programming.

According to the single-hit hypothesis of metabolic programming, exposure to single-hit of suboptimal nutrition immediately after birth can result in the manifestation of metabolic derangements in the individual either immediately or after a period of latency (Almond and Currie, 2011). The double-hit (two-hit hypothesis or multiple-hit hypothesis) hypothesis was first coined by cancer geneticist researcher, Dr. Alfred George Knudson in 1971 to address the multifactorial genetic interactions that lead to the development of cancer. This hypothesis has been adopted by researchers interrogating multifactorial interactions involved in the development metabolic programming. The double-hit hypothesis of metabolic programming states the 'first-hit' or 'single-hit' events in perinatal life alone are not sufficient to cause the onset of metabolic derangements in adult life (Tamashiro and Moran, 2010). It is argued that the 'first-hit' events merely increase the risk of developing metabolic derangements when a 'second-hit' multifactorial stimuli or factors are introduced in adulthood (Stewart *et al.*, 2013). Huynh *et al.* (2008) demonstrated that the 'first-hit' oral administration of 10% fructose solution to suckling Wistar rats increased susceptibility to developing metabolic dysfunction, obesity, and hyperinsulinemia following a 'second-hit' with 65% fructose w/w in the feed in adulthood.

The consumption of bitter melon powder (1% w/w in diet) by rats fed a high-fructose diet 'first-hit' during gestation and lactation protected their resultant offspring against a 'second-hit' high-fructose diet-induced metabolic derangements in adulthood. The protective effects of bitter melon powder against the adverse 'double-hit' high-fructose diet-induced metabolic programming noted by Ching *et al.* (2011) were ascribed to the bitter melon powder's antioxidant, antiobesity and antidiabetic properties. Other studies have also demonstrated that the maternal administration of antiobesity and antidiabetic substances during gestation and lactation protected offspring against the adverse outcomes of 'single-hit' and 'double-hit' diet-induced metabolic programming (Carvalho *et al.*, 2016; Desai *et al.*, 2013; Li *et al.*, 2012; Barbalho *et al.*, 2011; Elahi *et al.*, 2008). There is a dearth of studies that investigated and or investigate the potential of neonatal orally administered ethnomedicines with antiobesity and antidiabetic activity to protect against the adverse metabolic outcomes of 'early single-hit', 'late single-hit' and 'double-hit' diet-induced metabolic programming in adulthood.

S-allyl-cysteine (SAC), a water soluble organosulphur phytochemical constituent of garlic (Hoshino *et al.*, 2001) possesses antioxidant, antiobesity and antidiabetic properties which are

beneficial to health (Asdaq, 2015; Saravanan *et al.*, 2013; Saravanan and Ponmurugan, 2013). These beneficial effects of SAC suggest that it may be a viable intervention for protecting against the ‘early single-hit’, ‘late single-hit’ and ‘double-hit’ high-fructose diet-induced adverse metabolic derangements in adulthood. Nonetheless, there are no studies that have investigated the potential of neonatal orally administered SAC to protect against the adverse metabolic outcomes of early or late ‘single-hit’ and ‘double-hit’ high-fructose diet in adulthood. For the purposes of the current study ‘early single-hit’ high-fructose diet refers to neonatal orally administered 20% fructose solution, ‘late single-hit’ high fructose diet refers to consumption of 20% fructose drinking water in adulthood and ‘double-hit’ high-fructose diet refers to a combination of neonatal orally administered 20% fructose solution (early fructose-hit) and 20% fructose drinking water in adulthood (late fructose-hit). This study sought to determine the potential of orally administered SAC to suckling Wistar rats to protect against the development of metabolic derangements in adulthood when the rats were challenged by a “second hit” of a high-fructose diet in adulthood. The study specifically sought to determine the effects on growth performance (body mass and linear growth), blood metabolites (glucose, triglycerides, cholesterol and insulin concentration), liver lipid content, viscera morphometry and surrogate markers of liver (alanine aminotransferase and alkaline phosphatase) and kidney (creatinine and blood urea nitrogen) function.

Hypothesis

H₁: Oral neonatal administration of SAC protects adult male and female Wistar rats against the development metabolic derangements induced by either neonatal or adult “single hit” and or a combinatorial neonatal and later adult “double hit” high fructose diet.

H₀: Oral neonatal administration of SAC does not protect adult male and female Wistar rats against the development of metabolic derangements induced by either neonatal or adult “single hit” and or a combinatorial neonatal and later adult “double hit” high fructose diet.

4.1 Materials and methods

4.1.1 Animals, feeding and Housing

One hundred and twenty-eight, four-day-old, male and female Wistar rat pups from 12 first-time female breeders (litter size: minimum 8, maximum 12) were used in the study. The litter

size range used in the study was based studies that have shown that litter sizes that are too small or too large result in over nourishment and under undernourishment respectively, which alters metabolic function (Habbout *et al.*, 2013; Patterson *et al.*, 2010). The study was done in three experimental stages: experimental stage 1 was from postnatal day 6 to 20; experimental stage 2 was from postnatal day 21 to 55, and stage 3 was from postnatal day 56 to 116. During the first experimental stage, the rat pups in the litters were housed with their respective dams in Perspex cages lined with wood shavings in the Central Animal Services (CAS). After weaning (postnatal day 21 to onwards) the rats (weanling) were housed individually in Perspex cages. Room temperatures were maintained 24 ± 2 °C with a 12-h light/dark cycle (lights on from 07:00 h to 19:00 h) for all experimental stages. The rats had *ad libitum* access to standard rat chow pellets during the second experimental stage.

4.1.2 Experimental design

The first experimental stage, which was the neonatal intervention stage, was designed as described in chapter 3, subheading 3.1.4. Briefly, the pups were randomly allocated to and administered one of the following treatment regimens: group I - 10 ml/kg body mass per day of distilled water (DH), group II - 10 ml/kg body mass per day of 20% fructose solution (w/v) [FS], group III - 150 mg/kg body mass per day of S-allyl-cysteine dissolved in distilled water (SAC), group IV - 150 mg/kg body mass per day of SAC dissolved in distilled water and 10 ml/kg body mass per day of 20% fructose solution (w/v) [SAC + FS]. The random allocation was such that the male to female rat ratio was close to 1:1 as possible per treatment regimen and respective treatment regimens were administered daily to the respective rat pups for 15 days (postnatal day 6 to 20).

During the second experimental stage, which was the growth stage (postnatal day 22 to 55), the rats were weaned (postnatal day 21) and housed individually and their mothers returned to stock. In the second experimental stage, all rats were allowed *ad libitum* access to standard rat feed and plain drinking water.

The third experimental stage, which was the adulthood intervention stage, was conducted from postnatal 56 to 116. On postnatal day 56, the male and female rats from each treatment regimen during stage 1 were randomly allocated to two subgroups: subgroup I: continued on a standard rat chow (*ad libitum*) and plain drinking water (PDW) and sub-group II: standard

rat chow (*ad libitum*) and 20% fructose (w/v) drinking water (high-fructose diet group, FW). The rats in each of the subgroups were kept on their respective treatment regimens for 8 weeks and thereafter fasted overnight and then subjected to an oral glucose tolerance test and then returned on their respective treatment regimens and then euthanised 48 hours later.

Study design

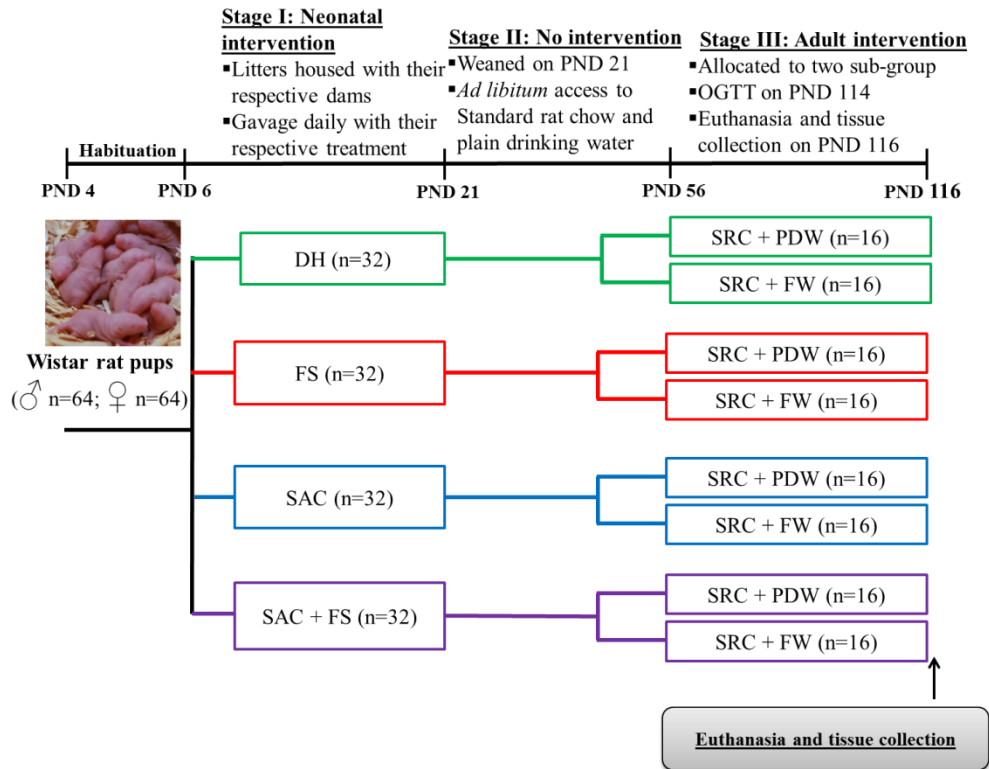


Figure 4.1: A schematic representation of the study design

DH = 10 ml/kg body mass per day distilled water; FS = 10 ml/kg body mass per day 20% fructose solution (w/v); SAC = 150 mg/kg body mass per day S-allyl-cysteine; SAC + FS = 150 mg/kg body mass per day S-allyl-cysteine + 10 ml/kg body mass per day 20% fructose solution (w/v); SRC + PDW = standard rat chow + plain drinking water; SRC + FW = standard rat chow + fructose drinking water; OGTT = oral glucose tolerance test; PND = postnatal day.

4.1.3 Measurements

4.1.3.1 Body mass

The rats were weighed daily in the first experimental stage (postnatal days 6 to 21) and twice a week during the second and third experimental stages (from postnatal day 22 to 55 and from postnatal day 56 to 116, respectively). An electronic balance (Snowrex Electronic Scale, Clover Scales, Johannesburg) was used to weigh the rats.

4.1.3.2 Oral glucose tolerance test

After 8 weeks (postnatal day 56 to 114) of high-fructose diet intervention during adulthood, the rats were fasted overnight and fasting blood glucose concentration determined from a drop of blood obtained from the tail vein via pinprick (Loxham *et al.*, 2007). The blood glucose concentration was determined using a calibrated Contour Plus glucometer (Bayer (Pty) LTD, Isando, South Africa) as per the manufacturer's instructions. Thereafter the rats were gavaged with 2 g/kg glucose (Kannappan and Anuradha, 2009) and blood collected from the tail vein to determine glucose concentration post-gavage at fixed time intervals (15, 30, 60 and 120 minutes using a calibrated glucose meter. The rats were put back on their respective treatment regimens for 48 hours to allow them to recover.

4.1.4 Terminal procedures

4.1.4.1 Determination of terminal body mass and fasted blood glucose

At the end of the third experimental stage (postnatal day 116), the rats were fasted overnight and then their terminal body masses were measured by placing them in a pre-weighed box on an electronic scale (Snowrex Electronic Scale, Clover Scales, Johannesburg). The rats were then euthanized by intraperitoneal injection of an overdose of sodium pentobarbitone (Euthanaze, Bayer (Pty) LTD, Isando, South Africa) at 200 mg/kg body mass.

4.1.4.2 Blood collection, processing, and storage

Following euthanasia, blood was collected via cardiac puncture using 18 G needles and 10 ml syringes into 6 ml heparinised blood collection tubes (Becton Dickinson Vacutainer Systems Europe, Meylan Cedex, France) and then spun for 15 min at 5000 g at 22 °C in a Senova NovaFuge centrifuge (Senova Labtech Co, LTD, Shanghai, China). Plasma was then collected and then stored at -20 °C for determination of triglycerides, cholesterol, and insulin concentrations and surrogate markers of liver (ALT and ALP) and kidney (creatinine and blood urea nitrogen) function.

4.1.4.3 Determination of visceral organ morphometry

Visceral organ morphometry was determined as described in chapter 3 under subheading 3.1.6.3. Additionally the visceral (mesenteric and perirenal) fat and epididymal fat (in males only) were dissected out and their masses determined by placing them in a pre-weighed weighing boat on an electronic scale (Presica 310M, Presica Instruments AG, Switzerland).

4.1.4.4 Determination of liver histo-morphometry

Liver histomorphometry and scoring was done as described in chapter 3 under subheading 3.1.6.4

4.1.5 Determination of fasting plasma triglycerides and cholesterol

Fasted plasma triglyceride concentration was determined using a calibrated Accutrend triglyceride meter (Roche, Mannheim, Germany) as per the manufacturer's instructions.

The fasted plasma cholesterol concentration was determined using a colorimetric-based clinical chemistry analyser (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., USA) as described in chapter 3 under subheading 3.19.

4.1.6 Determination of hepatic lipid content

The liver lipid content was determined as described chapter 3 under subheading 3.1.7.

4.1.7 Determination of plasma insulin concentration

The determination of plasma insulin concentration and computation of HOMA-IR were done as described in chapter 3 under subheading 3.1.8.

4.1.8 Determination of linear growth

The evaluation of morphometric (masses and lengths) parameters of tibiae, computation of Seedor indices and capturing of radiograph images were done as described in chapter 3 under subheading 3.1.9.

4.1.9 Determination of surrogate markers of liver and kidney function

The determination of the plasma ALT, ALP and amylase activity and plasma blood urea nitrogen and creatinine concentration was done using a colorimetric-based clinical chemistry analyser (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., USA) as described in chapter 3 under subheading 3.1.10.

4.1.10 Statistical analysis

Parametric data are expressed as mean \pm SD and non-parametric data are expressed as median and range (min, max). The data were analysed using GraphPad Prism 5 software (Graph-Pad Software Inc., San Diego, CA, USA). Statistical significance was considered when $p \leq 0.05$. Data on body mass and oral glucose tolerance test were analysed using a repeated measures analysis of variance (ANOVA). A one-way ANOVA was used to analyse other multiple group data followed by mean comparison using a Bonferroni *post hoc* test to compare means. The Kruskal-Wallis test (non-parametric one-way ANOVA) was used to

analyse non-alcoholic fatty liver disease score (NAS) multiple group data followed by Dunns *post hoc* test to compare medians.

The model used for the analysis of variance for weekly body masses and the glucose tolerance test was:

$$Y_{ijk} = \mu + T_i + B_j + C_k + e_{ijk}; \text{ where;}$$

Y_{ijk} = weekly body mass and or blood glucose concentration at time C post gavage

μ = overall mean common to all observations

C_k = fixed effect of sampling time on blood glucose concentration (= 1,2.....5)

T_i = effect of treatment regimen (n = 1,2.....8)

B_j = fixed effect of individual rat (1,2,3.....64)

e_{ijk} = residual random error

The model used for ANOVA for variables determined at study termination was:

$$Y_{ijk} = \mu + T_i + B_j + e_{ijk}; \text{ where;}$$

Y_{ijk} = response variable of interest

μ = overall mean to all observations

T_i = effect of treatment (n = 1,2,..8)

B_j = fixed effect of individual rat (1,2,3.....64)

e_{ijk} = residual random error

4.2 Results

4.2.1 Growth performance

4.2.1.1 Body mass

Figure 4.2 below shows the induction, weaning, and terminal body masses of male rats across the treatment regimens.

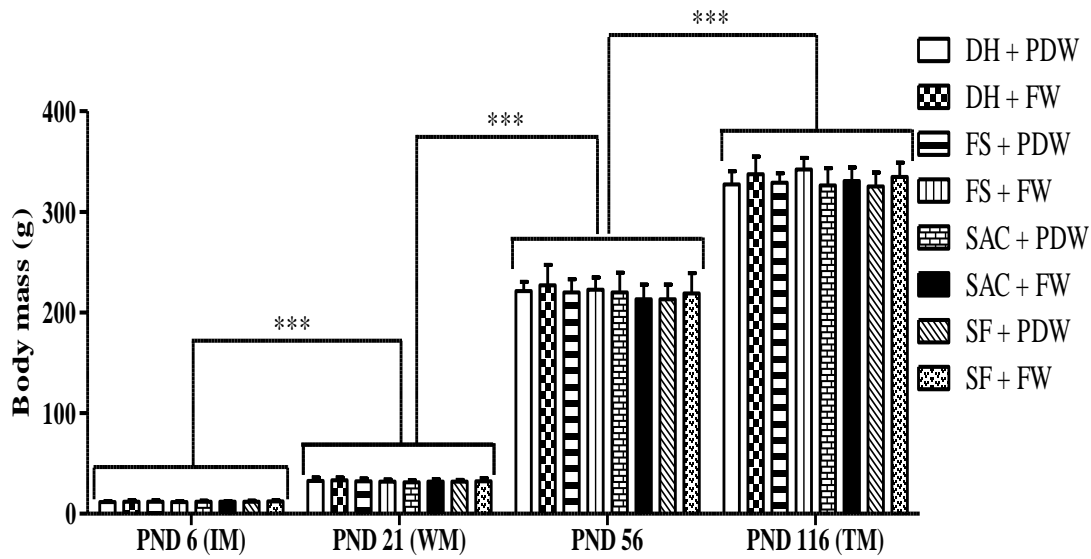


Figure 4.2: Effect of high-fructose diet on body masses of the male rat pups orally administered S-allyl-cysteine during suckling.

*** $p < 0.0001$. Induction, weaning, PND 56, and terminal body masses of the rats were similar across treatment regimens. The rats grew ($p < 0.0001$) significantly compared to their induction and weaning masses. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; IM = induction mass; PND = postnatal day; TM = terminal mass; WM = weaning mass. Data presented as mean \pm SD; $n = 8-9$ per treatment regimen.

There were no difference in the male rats' body masses at induction, weaning, PND 56 and termination across treatment regimens. However, across treatment regimens, the male rats grew significantly ($p < 0.0001$) from induction to weaning, from weaning to PND 56 and from PND 56 to termination of the study (Figure 4.2).

Figure 4.3 shows the induction, weaning and terminal body masses of female rats across treatment regimens.

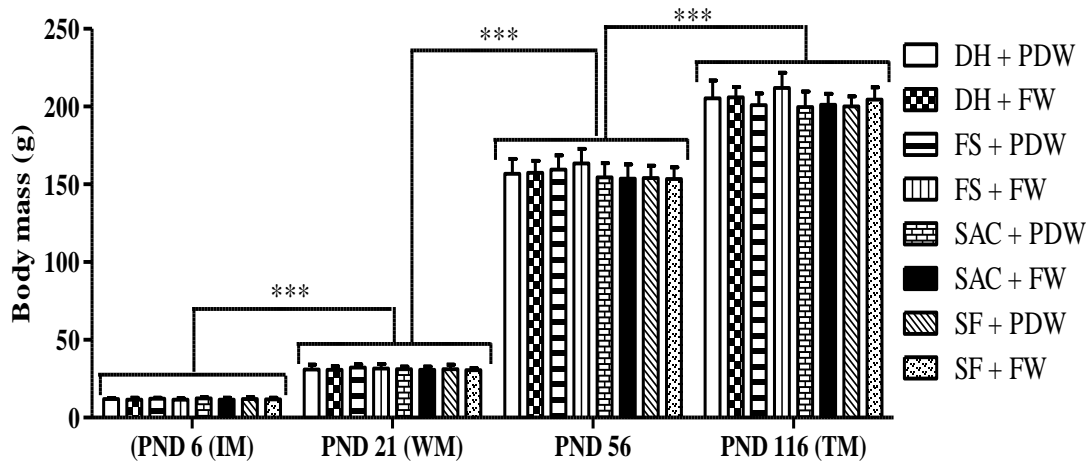


Figure 4.3: Effect of high-fructose diet on body masses of the female rat pups orally administered S-allyl-cysteine during suckling.

*** $p < 0.0001$. Induction, weaning, PND 56, and terminal body masses were similar across all treatment regimens. The rats grew ($p < 0.0001$) significantly compared to their induction and weaning masses. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; IM = induction mass; PND = postnatal day; TM = terminal mass; WM = weaning mass. Data presented as mean \pm SD; $n = 7-9$ per treatment.

There were no difference in female rats' body masses at induction, weaning, PND 56 and termination across treatment regimens. However, across treatment regimens, the female rats grew significantly ($p < 0.0001$) from induction to weaning, from weaning to PND 56 and from PND 56 to termination of the study (Figure 4.3).

4.2.1.2 Linear growth

Table 4.1 below shows lengths, masses and Seedor indices of tibiae of male rats following oral administration of their respective treatment regimens.

Table 4.1: Effects of high-fructose diet on tibia masses, lengths and Seedor indices of adult male rats orally administered S-allyl-cysteine during suckling

Treatments	Tibia mass (mg)	Tibia length (mm)	Tibia Seedor index (mg/mm)
DH + PDW	342.60 ± 17.04	39.89 ± 0.74	8.58 ± 0.42
DH + FW	337.60 ± 15.93	39.60 ± 0.77	8.53 ± 0.40
FS + PDW	337.80 ± 12.94	39.41 ± 1.54	8.58 ± 0.52
FS + FW	326.20 ± 14.13	39.69 ± 0.76	8.22 ± 0.27
SAC + PDW	338.30 ± 22.08	39.66 ± 0.74	8.53 ± 0.61
SAC + FW	341.80 ± 23.38	38.62 ± 3.40	8.88 ± 0.70
SF + PDW	346.10 ± 27.66	39.46 ± 0.56	8.78 ± 0.75
SF + FW	337.80 ± 14.62	39.23 ± 1.13	8.62 ± 0.46

Tibiae lengths, masses, and densities (Seedor indices) from rats across treatment regimens were similar. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood. Data presented as mean ± SD; n = 8-9 per treatment.

There were no differences in tibiae masses, lengths and densities of male rats across treatment regimens (Table 4.1).

Table 4.2 below shows lengths, masses and Seedor indices of tibiae of female rats following oral administration of their respective treatment regimens

Table 4.2: Effects of high-fructose diet on tibia masses, lengths and densities (Seedor indices) of adult female rats orally administered S-allyl-cysteine during suckling

Treatments	Tibia mass (mg)	Tibia length (mm)	Tibia Seedor index (mg/mm)
DH + PDW	289.30 ± 7.80	37.06 ± 1.33	7.82 ± 0.33
DH + FW	269.10 ± 17.11	36.30 ± 0.95	7.43 ± 0.62
FS + PDW	286.80 ± 7.42	36.01 ± 0.42	7.80 ± 0.28
FS + FW	284.60 ± 11.46	36.55 ± 1.39	7.80 ± 0.43
SAC + PDW	286.80 ± 9.53	35.77 ± 0.42	8.02 ± 0.23
SAC + FW	268.90 ± 33.50	36.66 ± 1.47	7.35 ± 1.02
SF + PDW	279.40 ± 13.33	35.58 ± 0.92	7.95 ± 0.51
SF + FW	287.40 ± 16.90	35.42 ± 0.56	8.11 ± 0.42

Tibiae lengths, masses, and densities (Seedor indices) from rats across treatment regimens were similar. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood. Data presented as mean ± SD; n = 7-9 per treatment.

There were no differences in the tibiae masses, lengths and densities of the female rats across treatment regimens (Table 4.2).

Figure 4.4 below shows the radiograph images of representative tibiae from male rats across treatment regimens.

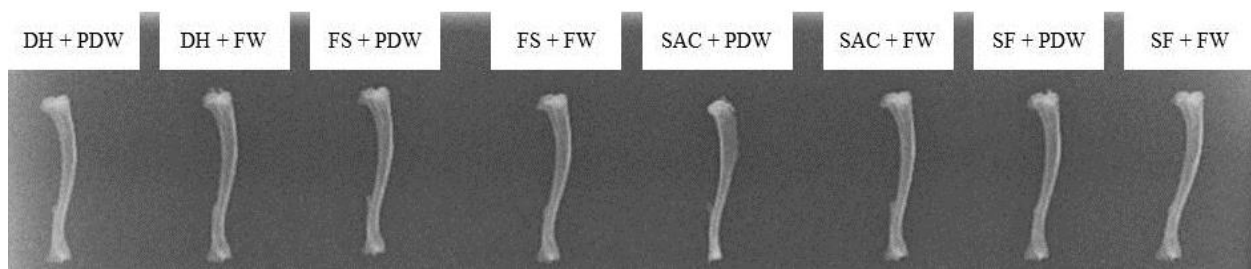


Figure 4.4: Tibiae radiograph images of adult male rats.

DH + PDW = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood.

There were no observable differences in the radiographical densities of tibiae from male rats across treatment regimens (Figure 4.4).

Figure 4.5 below shows the radiograph images of representative tibiae from female rats across treatment regimens.

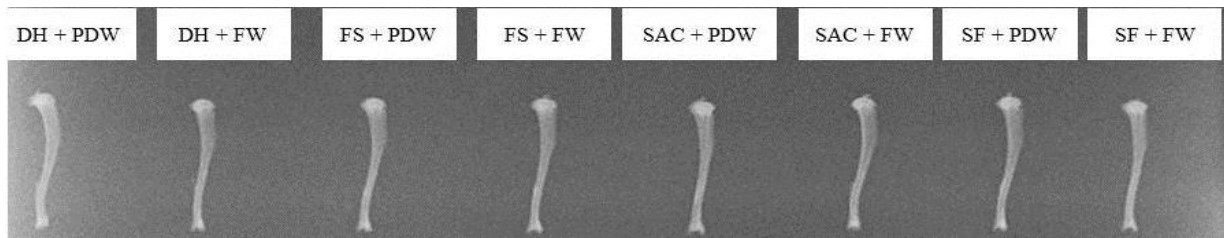


Figure 4.5: Tibiae radiograph images of adult female rats.

DH + PDW = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood.

There were no observable differences in the radiographical densities of tibiae from female rats across treatment regimens (Figure 4.5).

4.2.2 Glucose handling

4.2.2.1 Oral glucose tolerance test

Figure 4.6 shows the blood glucose concentration at basal/pre-gavage (0 min) and at 15, 30, 60 and 120 minutes post-gavage with 50% percent glucose solution at 2 g/kg body mass of male rats following 8 weeks on their respective treatment regimens.

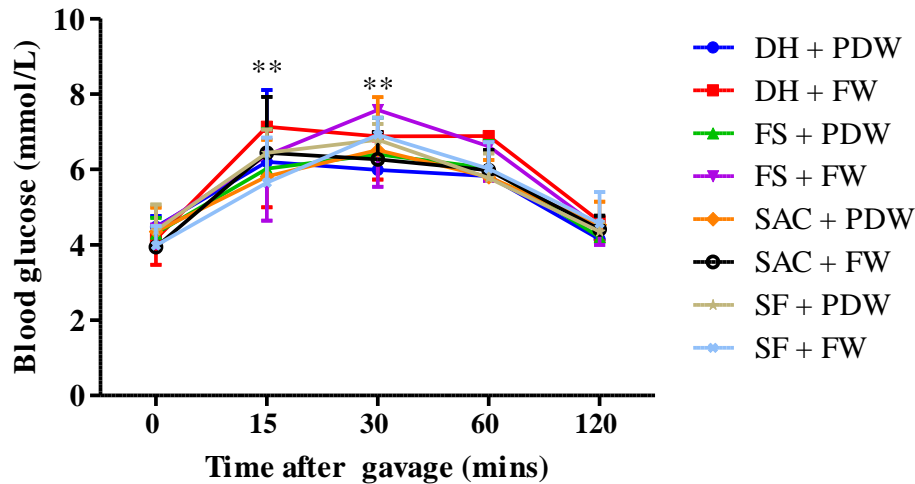


Figure 4.6: Effects of high-fructose diet on glucose tolerance of adult male rats orally administered S-allyl-cysteine during suckling.

** $p < 0.01$. Blood glucose concentration of rats administered DH + PDW, DH + FW and SAC + FW peaked 15 minutes post gavage and was significantly greater ($p < 0.01$) than the basal concentration and returned to basal concentration 120 minutes post-gavage. FS + PDW, FS + FW, SAC + PDW, SF + PDW and SF + FW rats' blood glucose concentration peaked 30 minutes post-gavage and was significantly greater ($p < 0.01$) than the basal concentration and returned to basal concentration 120 minutes post-gavage. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood. Data presented as mean \pm SD; n = 8-9 per treatment.

Male rats that were administered the following treatment regimens: neonatal oral administration of distilled water plus plain drinking water in adulthood (DH + PDW; control), neonatal oral administration of distilled water plus 20% fructose drinking water in adulthood (DH + FW) and neonatal oral administration of SAC plus 20% fructose drinking water in adulthood (SAC + FW) had significantly greater ($p < 0.01$) blood glucose concentration 15 minutes post-gavage compared to basal blood glucose concentration (Table 4.6). The blood glucose concentration of male rats administered the following treatment regimens: neonatal oral administration of 20% fructose solution plus plain drinking water in adulthood (FS + PDW), neonatal oral administration of 20% fructose solution plus 20% fructose drinking water in adulthood (FS + FW), neonatal oral administration of SAC plus plain drinking water in adulthood (SAC + PDW), neonatal oral administration of SAC and 20% fructose solution plus plain drinking water in adulthood (SF + PDW) and neonatal oral administration of SAC and 20% fructose solution plus 20% fructose drinking water in adulthood (SF + FW) was significantly higher ($p < 0.01$) than basal blood glucose concentration 30 minutes post-gavage (Table 4.6). The blood glucose concentration of male rats across treatment regimens returned to basal at 120 minutes post-gavage (Figure 4.6).

The blood glucose concentration of the female rats at time 0 (basal/pre-gavage), 15, 30, 60 and 120 minutes post-gavage with a 50% glucose solution at 2g/kg body mass following 8 weeks on their respective treatment regimens is shown in Figure 4.7.

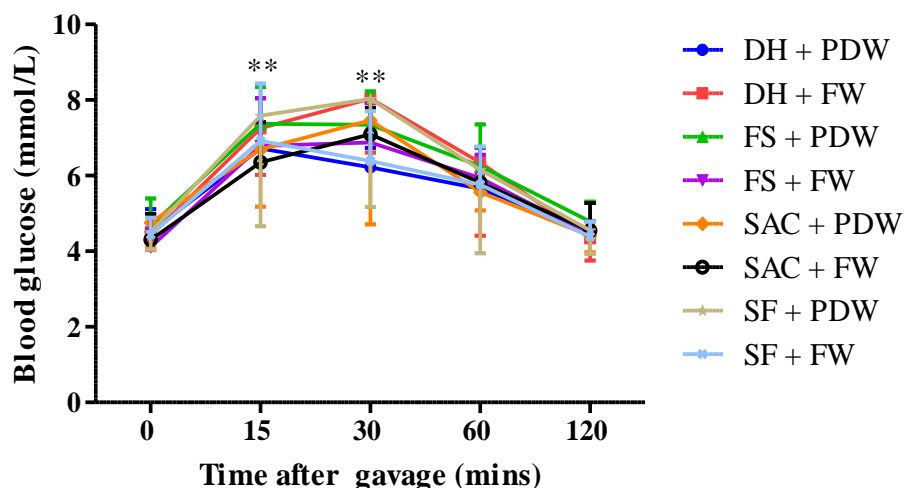


Figure 4.7: Effects of high-fructose diet on glucose tolerance of adult female rats orally administered S-allyl-cysteine during suckling.

** $p < 0.01$. Blood glucose concentration of rats administered DH + PDW, FS + PDW and SF + FW peaked 15 minutes post gavage and was significantly greater ($p < 0.01$) than the basal concentration and returned to basal concentration 120 minutes post-gavage. DH + FW, FS + FW, SAC + PDW, SAC + FW and SF + PDW rats' blood glucose concentration peaked 30 minutes post-gavage and were significantly greater ($p < 0.01$) than the basal concentration and returned to basal concentration 120 minutes post-gavage. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood. Data presented as mean \pm SD; $n = 7-9$ per treatment.

Female rats that were administered the following treatment regimens: neonatal oral administration of distilled water plus plain drinking water in adulthood (DH + PDW; control), neonatal oral administration of 20% fructose solution plus plain drinking water in adulthood (FS + PDW) and combined neonatal oral administration of SAC and 20% fructose solution plus 20% fructose drinking water in adulthood (SAC + FW) had significantly greater ($p < 0.01$) blood glucose concentration 15 minutes post-gavage compared to basal blood glucose concentration (Table 4.7). The blood glucose concentration of female rats administered the following treatment regimens: neonatal oral administration of distilled water plus 20% fructose drinking water in adulthood (DH + FW), neonatal oral administration of 20% fructose solution plus 20% fructose drinking water in adulthood (FS + FW), neonatal oral administration of SAC plus plain drinking water in adulthood (SAC + PDW), neonatal oral administration of SAC plus fructose drinking water in adulthood (SAC + FW), and neonatal oral administration of SAC and 20% fructose solution plus plain drinking water in adulthood (SF + PDW) was significantly higher ($p < 0.01$) than basal blood glucose concentration 30 minutes post-gavage (Table 4.7). The blood glucose concentration of female rats across treatment regimens returned to basal at 120 minutes post-gavage (Figure 4.7).

4.2.2. Area under the curve: oral glucose tolerance test

Figure 4.8 below shows the area under the curve calculated from the oral glucose tolerance test results of the male rats.

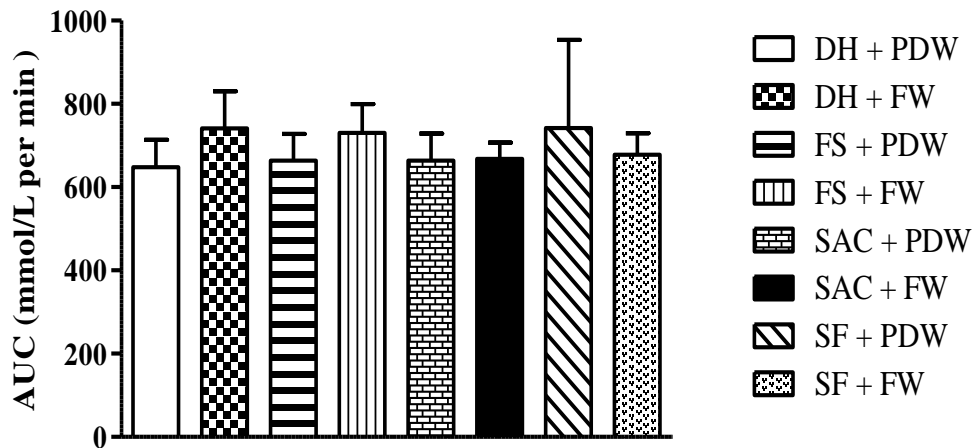


Figure 4.8: Effects of high-fructose diet on total area under the curve of oral glucose tolerance of adult male rats orally administered S-allyl-cysteine during suckling.

The total area under the curve of oral glucose tolerance of male rats was similar across treatment regimens. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood. Data presented as mean \pm SD; n = 8-9 per treatment.

There were no significant differences in total area under the curve of oral glucose tolerance of male rats across treatment regimens (Figure 4.8).

Figure 4.9 shows the area under the curve calculated from the oral glucose tolerance test results in the female rats.

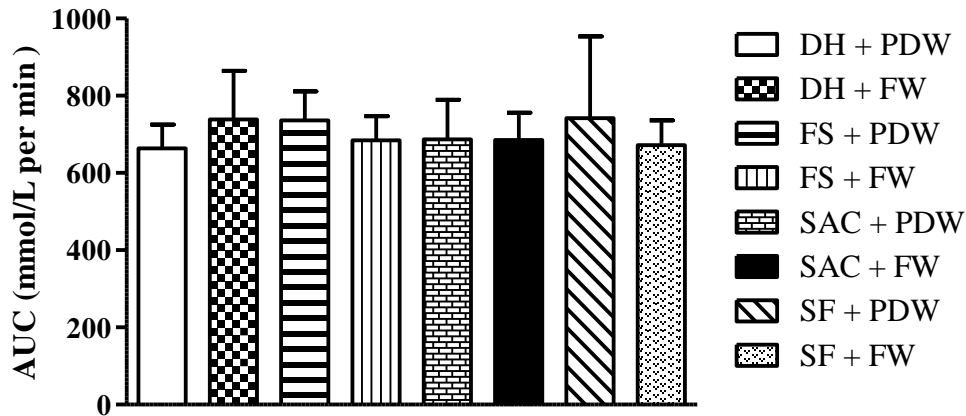


Figure 4.9: Effects of high-fructose diet on total area under the curve of oral glucose tolerance of adult female rats orally administered S-allyl-cysteine during suckling.

The total area under the curve of oral glucose tolerance of female rats was similar across treatment regimens. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood. Data presented as mean \pm SD; n = 7-9 per treatment.

There were no significant differences in total area under the curve of oral glucose tolerance of female rats across treatment regimens (Figure 4.9).

4.2.3 Blood parameters

4.2.3.1 Fasting blood glucose and plasma insulin concentration and HOMA-IR index

Table 4.3 shows the fasting blood glucose, plasma insulin concentrations and the HOMA-IR indices of the male rats after being administered their respective treatment regimens.

Table 4.3: Effects of high-fructose diet on fasting blood glucose, insulin and HOMA-IR indices of adult male rats orally administered S-allyl-cysteine during suckling

Treatments	Blood glucose (mmol/L)	Insulin ng/mL	HOMA-IR
DH + PDW	4.45 ± 0.30	21.01 ± 7.48	4.16 ± 1.50
DH + FW	4.15 ± 0.68	40.58 ± 22.05	6.97 ± 3.73
FS + PDW	4.30 ± 0.41	29.95 ± 20.15	5.90 ± 4.00
FS + FW	4.43 ± 0.51	35.47 ± 22.89	7.24 ± 5.11
SAC + PDW	4.35 ± 0.63	27.73 ± 13.49	5.17 ± 2.73
SAC + FW	3.94 ± 0.56	24.36 ± 17.59	4.18 ± 3.10
SF + PDW	4.38 ± 0.68	27.75 ± 7.38	5.13 ± 1.35
SF + FW	3.98 ± 0.52	11.52 ± 7.91	2.08 ± 1.46

Fasting blood glucose, plasma insulin and HOMA-IR were similar across treatment regimens. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; HOMA-IR = Homeostatic model of insulin resistance. Data presented as mean ± SD; n = 8-9 per treatment.

There were no significant differences in fasting blood glucose, plasma insulin and HOMA-IR of male rats across treatment regimens (Table 4.3).

Table 4.4 shows the fasting blood glucose, plasma insulin concentrations and HOMA-IR indices of female rats after being administered their respective treatment regimens.

Table 4.4: Effects of high-fructose diet on fasting blood glucose, insulin and HOMA-IR indices of adult female rats administered S-allyl-cysteine during suckling

Treatments	Blood glucose (mmol/L)	Insulin ng/mL	HOMA-IR
DH + PDW	4.52 ± 0.60	11.58 ± 7.27	2.28 ± 1.36
DH + FW	4.41 ± 0.38	32.47 ± 11.86	6.40 ± 2.58
FS + PDW	4.67 ± 0.72	17.11 ± 14.35	3.63 ± 3.62
FS + FW	4.11 ± 0.50	19.05 ± 15.60	3.27 ± 2.58
SAC + PDW	4.76 ± 0.67	16.67 ± 5.60	3.45 ± 0.75
SAC + FW	4.30 ± 0.68	15.49 ± 15.58	3.06 ± 3.33
SF + PDW	4.51 ± 0.47	22.73 ± 12.08	4.61 ± 2.70
SF + FW	4.44 ± 0.45	19.73 ± 15.34	4.14 ± 3.30

Fasting blood glucose, insulin and HOMA-IR were similar in female rats across all the treatment regimens. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; HOMA-IR = Homeostatic model of insulin resistance. Data presented as mean ± SD; n = 7-9 per treatment.

There were no significant differences in fasting blood glucose, plasma insulin and HOMA-IR of female rats across treatment regimens (Table 4.4).

4.2.3.2 Plasma triglyceride and cholesterol concentrations

Table 4.5 shows the plasma triglyceride and cholesterol concentrations in male rats following their respective treatment regimens.

Table 4.5: Effects of high-fructose diet on plasma triglyceride and cholesterol concentrations of adult male rats orally administered S-allyl-cysteine during suckling

Treatments	Triglyceride (mmol/L)	Cholesterol (mmol/L)
DH + PDW	1.71 ± 0.41	1.22 ± 0.51
DH + FW	1.70 ± 0.48	1.32 ± 0.48
FS + PDW	1.77 ± 0.57	1.60 ± 0.34
FS + FW	1.97 ± 0.64	1.30 ± 0.55
SAC + PDW	1.60 ± 0.83	1.30 ± 0.40
SAC + FW	1.87 ± 0.88	1.57 ± 0.73
SF + PDW	1.47 ± 0.40	1.35 ± 0.40
SF + FW	2.04 ± 0.41	1.10 ± 0.24

Plasma triglyceride and cholesterol concentrations of male rats were similar across treatment regimens. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood. Data presented as mean ± SD; n = 8-9 per treatment.

There were no significant differences in plasma triglyceride and cholesterol concentrations of male rats across treatment regimens (Table 4.5).

Table 4.6 shows the plasma triglyceride and cholesterol concentrations of female rats following their respective treatment regimens.

Table 4.6: Effects of high-fructose diet on plasma triglyceride and cholesterol concentrations of adult female rats orally administered S-allyl-cysteine during suckling

Treatments	Triglyceride (mmol/L)	Cholesterol (mmol/L)
DH + PDW	1.78 ± 0.78	1.47 ± 0.21
DH + FW	2.45 ± 0.73	1.26 ± 0.28
FS + PDW	1.67 ± 0.44	1.56 ± 0.30
FS + FW	1.99 ± 0.83	1.62 ± 0.28
SAC + PDW	1.70 ± 0.88	1.50 ± 0.33
SAC + FW	1.83 ± 0.52	1.30 ± 0.50
SF + PDW	1.47 ± 0.72	1.36 ± 0.27
SF + FW	2.07 ± 0.81	1.52 ± 0.21

Plasma triglyceride and cholesterol concentrations of female rats were similar across treatment regimens. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood. Data presented as mean ± SD; n = 7-9 per treatment.

There were no significant differences in the plasma triglyceride and cholesterol concentration of female rats across treatment regimens (Table 4.6).

4.2.4 Visceral organ morphometry

4.2.4.1 Small and large intestine lengths and masses

Table 4.7 shows the small and large intestine absolute and relative masses and lengths of male rats after being administered their respective treatment regimens.

Table 4.7: Effects of high-fructose diet on small and large intestine macro-morphometry of adult male rats orally administered S-allyl-cysteine during suckling

Treatments	SI (g)	SI TLr (g/mm)	SI (mm)	LI (g)	LI TLr (g/mm)	LI (mm)
DH + PDW	6.23 ± 0.58	0.16 ± 0.01	1163 ± 27.51	1.73 ± 0.22	0.04 ± 0.005	230.6 ± 12.86
DH + FW	6.31 ± 0.67	0.16 ± 0.02	1127 ± 91.45	1.70 ± 0.30	0.04 ± 0.007	223.3 ± 11.18
FS + PDW	6.44 ± 0.28	0.16 ± 0.01	1170 ± 35.46	1.85 ± 0.22	0.04 ± 0.005	225.0 ± 10.69
FS + FW	6.57 ± 0.49	0.16 ± 0.01	1212 ± 143.9	1.73 ± 0.27	0.04 ± 0.007	231.1 ± 9.280
SAC + PDW	6.48 ± 0.38	0.16 ± 0.01	1149 ± 16.91	1.78 ± 0.14	0.04 ± 0.003	232.8 ± 19.22
SAC + FW	6.53 ± 0.54	0.17 ± 0.02	1118 ± 38.24	1.66 ± 0.16	0.04 ± 0.005	222.2 ± 21.08
SF + PDW	6.24 ± 0.45	0.16 ± 0.01	1168 ± 30.23	1.70 ± 0.16	0.04 ± 0.004	232.5 ± 19.09
SF + FW	6.47 ± 0.62	0.16 ± 0.02	1067 ± 119.1	1.55 ± 0.09	0.04 ± 0.003	220.0 ± 8.944

Small and large intestine masses and lengths of male rats were similar across treatment regimens. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20%

fructose (w/v) in their drinking water in adulthood; LI = large intestine; SI = small intestine; TLr = relative to tibia length. Data presented as mean \pm SD; n = 8-9 per treatment.

There were no significant differences in small and large intestine masses and lengths from the male rats across treatment regimens (Table 4.7).

Table 4.8 shows the small and large intestine absolute and relative masses and lengths of female rats after being administered their respective treatment regimens.

Table 4.8: Effects of high-fructose diet on small and large intestine macro-morphometry of adult female rats orally administered S-allyl-cysteine during suckling

Treatments	SI (g)	SI TLr (g/mm)	SI (mm)	LI (g)	LI TLr (g/mm)	LI (mm)
DH + PDW	4.85 ± 0.60	0.13 ± 0.02	1070 ± 50.20	1.40 ± 0.09	0.04 ± 0.003	211.7 ± 27.87
DH + FW	4.90 ± 0.28	0.13 ± 0.01	1033 ± 29.63	1.25 ± 0.08	0.03 ± 0.002	207.5 ± 14.88
FS + PDW	4.85 ± 0.38	0.13 ± 0.01	1054 ± 22.64	1.41 ± 0.11	0.04 ± 0.003	206.9 ± 16.24
FS + FW	4.95 ± 0.35	0.13 ± 0.01	1058 ± 50.07	1.33 ± 0.14	0.03 ± 0.003	202.5 ± 8.864
SAC + PDW	4.91 ± 0.46	0.12 ± 0.01	1055 ± 36.25	1.40 ± 0.17	0.04 ± 0.004	196.3 ± 29.73
SAC + FW	4.75 ± 0.48	0.13 ± 0.01	1030 ± 30.55	1.22 ± 0.06	0.03 ± 0.003	204.3 ± 5.345
SF + PDW	4.82 ± 0.16	0.13 ± 0.01	1050 ± 29.44	1.32 ± 0.10	0.04 ± 0.002	210.7 ± 13.67
SF + FW	4.70 ± 0.25	0.13 ± 0.01	1036 ± 48.35	1.23 ± 0.13	0.03 ± 0.003	196.5 ± 13.75

Small and large intestine masses and lengths of male rats were similar across treatments. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20%

fructose (w/v) in their drinking water in adulthood; LI = large intestine; SI = small intestine; TLr = relative to tibia length. Data presented as mean \pm SD; n = 7-9 per treatment.

There were no significant differences in small and large intestine masses and lengths from female rats across treatment regimens (Table 4.8).

4.2.4.2 Visceral fat and epididymal fat pad masses

Table 4.9 shows the absolute and relative masses of visceral fat and epididymal fat masses from male rats after being administered their respective treatment regimens.

Table 4.9: Effects of high-fructose diet on visceral fat pad and epididymal fat pad masses of adult male rats orally administered S-allyl-cysteine during suckling

Treatments	VFP (g)	VFP TLR (g/mm)	EFP (g)	EFP TLR (g/mm)
DH + PDW	4.93 ± 0.93 ^a	0.12 ± 0.02 ^a	3.59 ± 0.59 ^a	0.09 ± 0.01 ^a
DH + FW	7.44 ± 0.99 ^b	0.20 ± 0.03 ^b	4.30 ± 0.93 ^b	0.11 ± 0.02 ^b
FS + PDW	5.26 ± 0.52 ^a	0.13 ± 0.01 ^a	3.32 ± 0.55 ^a	0.08 ± 0.01 ^a
FS + FW	7.67 ± 0.93 ^b	0.20 ± 0.03 ^b	4.77 ± 0.77 ^b	0.12 ± 0.02 ^b
SAC + PDW	5.70 ± 1.17 ^a	0.14 ± 0.03 ^a	3.78 ± 0.74 ^a	0.09 ± 0.02 ^a
SAC + FW	7.32 ± 1.18 ^b	0.20 ± 0.04 ^b	4.32 ± 0.70 ^b	0.12 ± 0.02 ^b
SF + PDW	5.56 ± 0.57 ^a	0.14 ± 0.01 ^a	3.41 ± 0.39 ^a	0.08 ± 0.01 ^a
SF + FW	8.17 ± 1.19 ^b	0.21 ± 0.03 ^b	4.92 ± 0.82 ^b	0.12 ± 0.02 ^b

^{ab}Within column means with different superscripts are significantly different at $p < 0.0001$.

Regardless of neonatal oral administration intervention, all male rats that consumed FW in adulthood had significantly heavier ($p < 0.05$) visceral and epididymal fat masses compared to all male rats that consumed PDW in adulthood. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; EFP = epididymal fat pad; TLR = relative to tibia length; VFP = visceral fat pad. Data presented as mean ± SD; n = 8-9 per treatment.

The consumption of 'late single-hit' high-fructose diet resulted in significantly heavier ($p < 0.0001$) visceral and epididymal fat masses in male rats (Table 4.9). Orally administered 'early single-hit' high-fructose diet did not predispose the male rats to have heavier visceral and epididymal fat masses in adulthood (Table 4.9). A 'double-hit' (neonatal orally administered 20% fructose solution followed by 20% fructose drinking water in adulthood) resulted in significantly increased ($p < 0.0001$) visceral and epididymal fat masses in male rats, but the increase in visceral and epididymal fat masses was not significantly different or higher to the 'late single-hit' high-fructose diet-induced increase (Table 4.9). Neonatal orally administered SAC did not prevent the 'late single-hit' or 'double-hit' (early and late) high-fructose diet-induced increase in visceral and epididymal fat masses (Table 4.9).

Table 4.10 shows the visceral fat pad absolute and relative masses of female rats after being administered their respective treatment regimens.

Table 4.10: Effects of high-fructose diet on visceral fat pad masses (absolute and relative to tibia length) of adult female rats orally administered S-allyl-cysteine during suckling

Treatments	VFP (g)	VFP TLR (g/mm)
DH + PDW	5.08 ± 1.20 ^a	0.14 ± 0.03 ^a
DH + FW	7.44 ± 0.65 ^b	0.20 ± 0.02 ^b
FS + PDW	5.18 ± 1.43 ^a	0.14 ± 0.04 ^a
FS + FW	8.80 ± 0.21 ^b	0.24 ± 0.05 ^b
SAC + PDW	5.13 ± 1.07 ^a	0.14 ± 0.03 ^a
SAC + FW	8.04 ± 0.88 ^b	0.22 ± 0.02 ^b
SF + PDW	5.06 ± 1.57 ^a	0.14 ± 0.04 ^a
SF + FW	8.18 ± 2.21 ^b	0.23 ± 0.06 ^b

^{ab}Within column means with different superscripts are significantly different at $p \leq 0.0001$.

Regardless of neonatal oral administration intervention, all female rats that consumed FW in adulthood had significantly heavier ($p < 0.05$) visceral and epididymal fat masses compared to all female rats that consumed PDW in adulthood. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; TLR = relative to tibia length; VFP = visceral fat pad. Data presented as mean ± SD; n = 7-9 per treatment.

The consumption of 'late single-hit' high-fructose diet resulted in significantly heavier ($p < 0.0001$) visceral fat mass in female rats (Table 4.10). Orally administered 'early single-hit' high-fructose diet did not predispose the female rats to have increased visceral fat mass in adulthood (Table 4.10). A 'double-hit' (neonatal orally administered 20% fructose solution plus 20% fructose drinking water in adulthood) also resulted in significantly heavier ($p < 0.0001$) visceral fat mass in female rats, but the increase in the visceral fat mass was not significantly different or higher to the 'late single-hit' high-fructose diet-induced increase (Table 4.10). Neonatal orally administered SAC did not prevent the 'late single-hit' or 'double-hit' (early and late) fructose-induced increase in visceral fat mass (Table 4.10).

4.2.4.3 Liver masses (absolute and relative) and total liver lipid content

Liver masses (absolute and relative) and total liver lipid content of male rats are shown in Table 4.11.

Table 4.11: Effects of high-fructose diet on liver masses (absolute and relative to tibia length) and total liver lipid content of adult male rats orally administered S-allyl-cysteine during suckling

Treatments	Liver (g)	Liver TLR (g/mm)	Total liver lipid (%)
DH + PDW	8.46 ± 0.87 ^a	0.21 ± 0.02 ^a	2.82 ± 0.09 ^a
DH + FW	9.53 ± 0.92 ^a	0.24 ± 0.02 ^a	2.81 ± 0.09 ^a
FS + PDW	9.30 ± 0.42 ^a	0.23 ± 0.01 ^a	4.05 ± 0.01 ^b
FS + FW	9.75 ± 0.84 ^a	0.24 ± 0.02 ^a	3.13 ± 0.03 ^c
SAC + PDW	8.99 ± 0.45 ^a	0.23 ± 0.01 ^a	3.01 ± 0.22 ^c
SAC + FW	9.82 ± 0.63 ^a	0.26 ± 0.02 ^a	2.80 ± 0.08 ^a
SF + PDW	9.14 ± 1.03 ^a	0.23 ± 0.03 ^a	2.60 ± 0.12 ^d
SF + FW	9.34 ± 0.53 ^a	0.24 ± 0.01 ^a	3.00 ± 0.04 ^c

^{abc}Within column means with different superscripts are significantly different at $p \leq 0.05$.

Liver masses were similar across treatment regimens. Liver lipid content was significantly higher ($p < 0.05$) in FS + PDW male rats compared to DH + PDW, DH + FW or FS + FW male rats. SAC + PDW male rats had significantly higher ($p < 0.05$) liver lipids compared to DH + PDW, DH + FW and SAC + FW male rats. Liver lipid was significantly lower ($p < 0.05$) in SF + PDW male rats compared to FS + PDW, FS + FW, SF + FW, DH + PDW or DH + FW male rats. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood. Data presented as mean ± SD; n = 8-9 per treatment.

Orally administered 'early single-hit' high-fructose diet resulted in significantly ($p < 0.05$) higher liver lipid content in adult male rats (Table 4.11). A 'double-hit' (early and late) high-fructose diet resulted in significantly increased ($p < 0.05$) liver lipid content in adult male rats, but the increase in liver lipid content was not significantly higher to the 'early single-hit' induced increase in liver lipid content (Table 4.11). The consumption of 'late single-hit' high-fructose diet did not result in increased liver lipid content in adult male rats (Table 4.11). Neonatal orally administered SAC protected against 'early single-hit' high-fructose diet-induced increase in liver lipid content but not protect against the 'double-hit' (early and late) high-fructose diet-induced increase in liver lipid content (Table 4.11). Neonatal orally administered SAC resulted in significantly increased ($p < 0.05$) liver lipid content in male rats (Table 4.11). A combination of neonatal orally administered SAC and 'late single-hit' high-fructose diet did not result in significantly increase liver lipid content in male rats (Table 4.11).

Liver masses (absolute and relative) and total liver lipid content of female rats are shown in Table 4.12.

Table 4.12: Effects of high-fructose diet on liver masses (absolute and relative to tibia length) and total liver lipid content of adult female rats orally administered S-allyl-cysteine during suckling

Treatments	Liver (g)	Liver TLR (g/mm)	Total liver lipid (%)
DH + PDW	5.45 ± 0.78 ^a	0.15 ± 0.02 ^a	2.83 ± 0.01 ^a
DH + FW	5.89 ± 0.57 ^a	0.16 ± 0.02 ^a	3.26 ± 0.24 ^b
FS + PDW	5.36 ± 0.53 ^a	0.15 ± 0.01 ^a	4.11 ± 0.03 ^c
FS + FW	5.83 ± 0.51 ^a	0.16 ± 0.01 ^a	2.80 ± 0.02 ^a
SAC + PDW	5.71 ± 0.70 ^a	0.16 ± 0.02 ^a	3.32 ± 0.03 ^b
SAC + FW	5.63 ± 0.52 ^a	0.15 ± 0.01 ^a	2.79 ± 0.07 ^a
SF + PDW	5.80 ± 0.55 ^a	0.16 ± 0.01 ^a	2.72 ± 0.01 ^a
SF + FW	5.72 ± 0.60 ^a	0.16 ± 0.02 ^a	2.97 ± 0.03 ^a

^{abcd} Within column means with different superscripts are significantly different at $p \leq 0.05$.

Liver masses were similar across treatment regimens. Liver lipid content was significantly higher ($p < 0.05$) in DH + FW female rats compared to DH + PDW female rats. FS + PDW female rats had significantly higher ($p < 0.05$) liver lipids compared to FS + FW, DH + PDW, SF + PDW or SF + FW female rats. Liver lipid content was significantly higher ($p < 0.05$) in SAC + PDW female rats compared to DH + PDW or SAC + FW female rats. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood. Data presented as mean ± SD; n = 7-9 per treatment.

Orally administered 'early single-hit' high-fructose diet resulted in significantly ($p < 0.05$) higher liver lipid content in adult female rats (Table 4.12). The consumption of 'late single-hit' high-fructose diet resulted in significantly increased ($p < 0.05$) liver lipid content in adult female rats, but the increase in liver lipid content was not significantly higher to the 'early single-hit' induced increase in liver lipid content (Table 4.12). A 'double-hit' (early and late) high-fructose diet did not result in significantly increased liver lipid content in adult female rats (Table 4.12). Neonatal orally administered SAC protected the female rats against 'early single-hit' high-fructose diet and or 'late single-hit' high-fructose diet-induced increases in liver lipid content (Table 4.12). Neonatal orally administered SAC resulted in significantly increased ($p < 0.05$) liver lipid content in female rats (Table 4.12). A combination of neonatal orally administered SAC and 'late single-hit' high-fructose diet did not result in significantly increased liver lipid content in female rats (Table 4.12).

4.2.4.4 Liver histo-morphometry

Table 4.13 shows the hepatocyte cell size and numbers of hepatocytes of male rats after being administered their respective treatment regimens.

Table 4.13: Effects of high-fructose diet on the size and density of hepatocytes from adult male rats orally administered S-allyl-cysteine during suckling

Treatments	Hepatocyte size (μm)	Hepatocyte density (cells per 100 μm)
DH + PDW	9.83 \pm 1.61	10.16 \pm 0.76
DH + FW	9.83 \pm 1.15	10.00 \pm 1.00
FS + PDW	9.66 \pm 1.53	10.16 \pm 0.28
FS + FW	9.67 \pm 0.58	10.33 \pm 0.57
SAC + PDW	9.16 \pm 1.25	10.67 \pm 1.26
SAC + FW	9.67 \pm 0.30	10.67 \pm 0.76
SF + PDW	9.50 \pm 0.5	11.00 \pm 1.00
SF + FW	9.67 \pm 0.28	10.17 \pm 0.57

Hepatocyte size and density of male rats were similar across treatment regimens. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood. Data presented as mean \pm SD; n = 3 per treatment.

There were no significant differences in hepatocyte size and density from male rats across treatment regimens (Table 4.13).

Table 4.14 shows the hepatocyte cell size and density of hepatocytes of female rats after being administered their respective treatment regimens.

Table 4.14: Effects of high-fructose diet on the size and density of hepatocytes from adult female rats orally administered S-allyl-cysteine during suckling

Treatments	Hepatocyte size (μm)	Hepatocytes (cells per 100 μm)
DH + PDW	8.00 \pm 0.86	13.00 \pm 0.50
DH + FW	8.33 \pm 0.76	12.00 \pm 0.50
FS + PDW	8.00 \pm 0.50	12.00 \pm 0.86
FS + FW	8.17 \pm 1.04	12.00 \pm 1.32
SAC + PDW	8.17 \pm 0.28	13.00 \pm 0.50
SAC + FW	8.33 \pm 1.04	12.67 \pm 0.28
SF + PDW	8.50 \pm 0.86	12.00 \pm 1.32
SF + FW	8.17 \pm 0.76	12.67 \pm 1.26

Hepatocyte size and density of female rats were similar across treatment regimens. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood. Data presented as mean \pm SD; n = 3 per treatment.

There were no significant differences in hepatocyte size and density of female rats across treatment regimens (Table 4.14).

Table 4.15 shows non-alcoholic fatty liver disease activity score (NAS) of male rats after being administered their respective treatment regimen.

Table 4.15: Effects of high-fructose diet on non-alcoholic fatty liver disease activity score (NAS) of adult male rats orally administered S-allyl-cysteine during suckling

Treatments	Steatosis score	Ballooning score	Lobular inflammation score	Total NAS
DH + PDW	0 (0, 0)	0 (0, 0)	2.5 (0, 3)	2.5 (0, 3)
DH + FW	0 (0, 1)	0 (0, 1)	2 (2, 3)	2.5 (2, 4)
FS + PDW	0 (0, 2)	0 (0, 0)	2 (1, 2)	2 (1, 4)
FS + FW	0 (0, 2)	0 (0, 0)	2 (2, 3)	3 (2, 5)
SAC + PDW	0 (0, 0)	0 (0, 0)	2 (2, 3)	2 (2, 3)
SAC + FW	0 (0, 0)	0 (0, 0)	2 (1, 3)	2 (1, 3)
SF + PDW	0 (0, 0)	0 (0, 1)	2 (1, 3)	2 (2, 3)
SF + FW	0 (0, 1)	0 (0, 1)	2 (0, 3)	2.5 (0, 5)

Non-alcoholic fatty liver disease activity scores (NAS) of male rats were similar across treatment regimens. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; NAS = non-alcoholic fatty liver disease activity score. Total NAS is the sum of values recorded for each category. Total NAS score interpretation: <2 = not steatohepatitis; 3–4 = uncertain; >5 = probable or definite steatohepatitis. Data presented as median and range (min; max); n = 4-5 per treatment.

The treatment regimens had no significant effect on non-alcoholic fatty liver disease activity score (NAS) of male rats (Table 4.15).

Table 4.16 shows non-alcoholic fatty liver disease activity score (NAS) of female rats after being administered their respective treatment regimens.

Table 4.16: Effects of high-fructose diet on non-alcoholic fatty liver disease activity score (NAS) of adult female rats orally administered S-allyl-cysteine during suckling

Treatments	Steatosis score	Ballooning score	Lobular inflammation score	Total NAS
DH + PDW	0 (0, 0)	0 (0, 0)	3 (2, 3)	3 (2, 3)
DH + FW	0 (0, 1)	0 (0, 1)	2 (1, 3)	2 (1, 5)
FS + PDW	1 (0, 2)	0 (0, 0)	2 (2, 2)	3 (2, 4)
FS + FW	0 (0, 2)	0 (0, 0)	2 (2, 2)	2 (2, 4)
SAC + PDW	0 (0, 1)	0 (0, 1)	3 (1, 3)	3.5 (1, 4)
SAC + FW	1 (0, 0)	0 (0, 0)	2 (1, 3)	3 (1, 4)
SF + PDW	0 (0, 1)	0 (0, 1)	2 (1, 2)	2 (1, 3)
SF + FW	0 (0, 0)	0 (0, 1)	2 (1, 3)	2.5 (1, 3)

Non-alcoholic fatty liver disease activity scores (NAS) of female rats were similar across treatment regimens. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; NAS = non-alcoholic fatty liver disease activity score. Total NAS is the sum of values recorded for each category. Total NAS score interpretation: <2 = not steatohepatitis; 3–4 = uncertain; >5 = probable or definite steatohepatitis. Data presented as median and range (min, max); n = 4-5 per treatment.

The treatment regimens had no significant effect on non-alcoholic fatty liver disease activity score (NAS) of female rats (Table 4.16).

Representative liver histology photo sections (H and E staining, 400 X magnification) of adult male rat from the different treatment groups are shown in Figure 4.10 below.

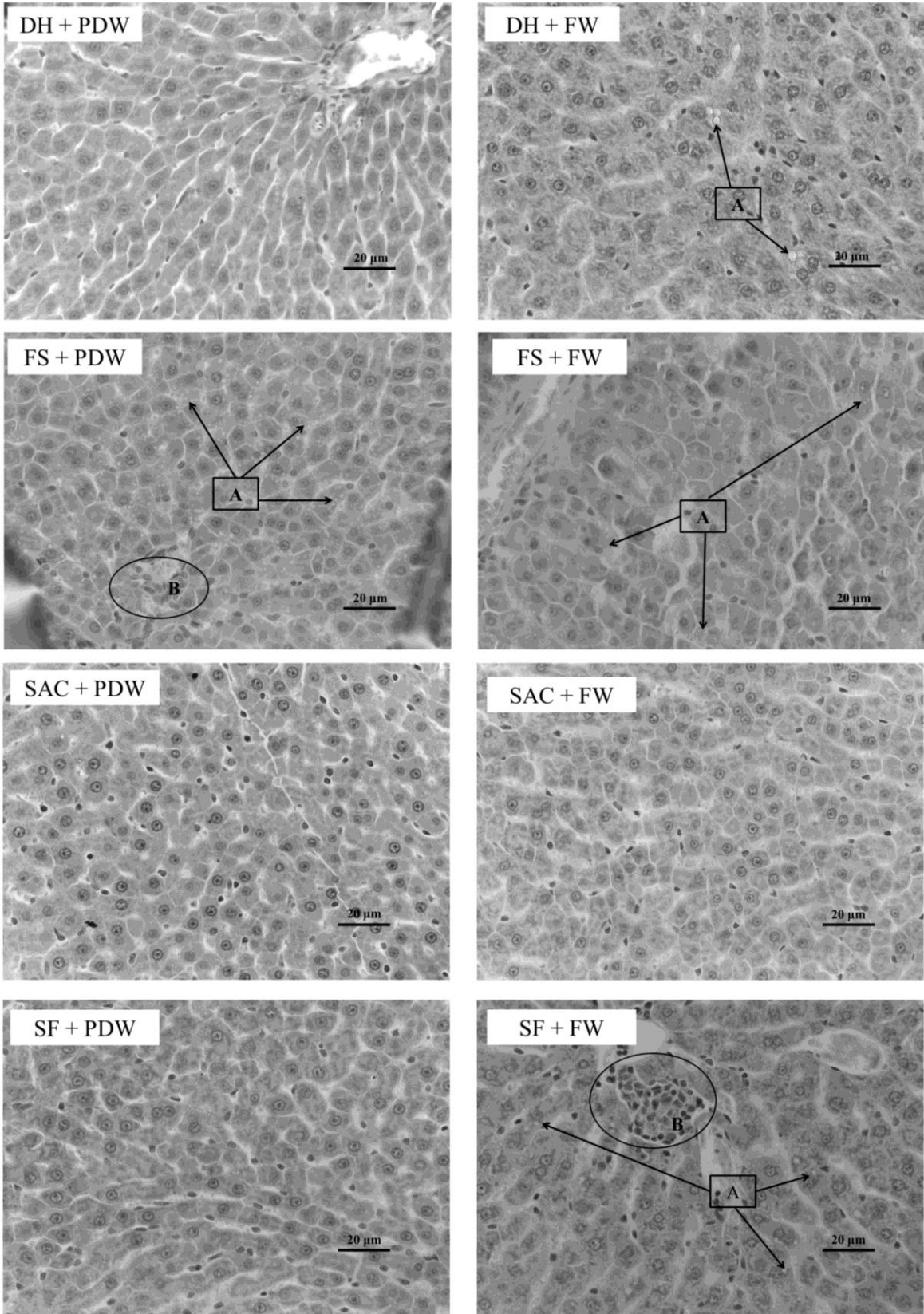


Figure 4.10: Effects of high-fructose diet on liver histology (H and E staining, 400 X magnification) of representative adult male rats orally administered S-allyl-cysteine during suckling.

Arrows A point to hepatic microvesicular steatosis and circle B shows foci of lobular inflammation. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood.

'Early single-hit', 'late single-hit' and 'double-hit' (early and late) high-fructose diet resulted in the development of microvesicular steatosis in male rats (Figure 4.10). Orally administered neonatal SAC prevented 'early single-hit' and 'late single-hit' high-fructose diet-induced microvesicular in male rats but did not prevent and 'double-hit' (early and late) high-fructose diet-induced microvesicular steatosis (Figure 4.10). Although neonatal orally administered SAC alone increased liver lipid content (Table 4.11) in male rats, it did not cause microvesicular steatosis (Figure 4.10).

Representative liver histology photo sections (H and E staining, 400 X magnification) of adult female rat from the different treatment groups are shown in Figure 4.11 below.

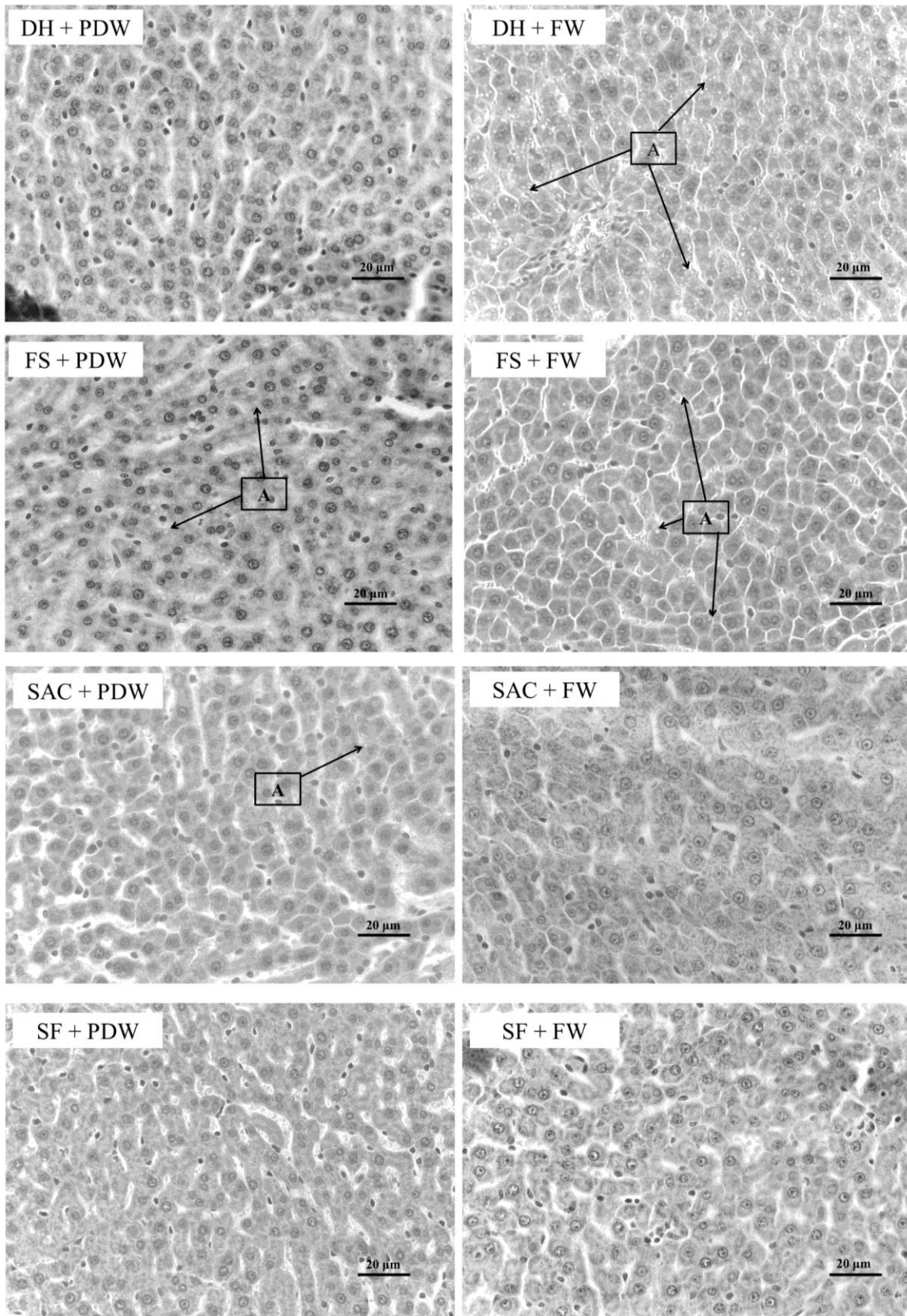


Figure 4.11: Effects of high-fructose diet on liver histology (H and E staining, 400 X magnification) of representative adult female rats orally administered S-allyl-cysteine during suckling.

Arrows A point to hepatic microvesicular steatosis. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood.

The 'early single-hit', 'late single-hit' and 'double-hit' (early and late) high-fructose diets resulted in the development of microvesicular steatosis in female rats (Figure 4.11). Orally administered neonatal SAC prevented 'early single-hit', 'late single-hit' and 'double-hit' (early and late) high-fructose diet-induced microvesicular in female rats (Figure 4.11). The neonatal orally administered SAC alone resulted in microvesicular steatosis (Figure 4.11).

4.2.5 Surrogate biomarkers of liver and kidney function

Table 4.17 shows the plasma activities of alanine aminotransferase and alanine phosphatase and plasma concentrations creatinine and blood urea nitrogen of male rats following their respective treatment regimens.

Table 4.17: Effects of high-fructose diet on surrogate markers of liver and kidney function of adult male rats orally administered S-allyl-cysteine during suckling

Treatments	ALT (U/L)	ALP (U/L)	BUN (mg/dL)	Creatinine (mg/dL)
DH + PDW	51.00 ± 9.35	63.33 ± 19.99	7.59 ± 0.83	34.22 ± 5.04
DH + FW	51.78 ± 8.01	66.22 ± 9.22	6.25 ± 0.48	37.11 ± 5.77
FS + PDW	54.67 ± 8.17	88.00 ± 14.78	7.20 ± 0.78	35.22 ± 7.51
FS + FW	56.67 ± 12.07	69.33 ± 19.84	6.48 ± 0.68	38.11 ± 6.133
SAC + PDW	65.22 ± 14.75	81.00 ± 14.20	7.30 ± 1.07	38.11 ± 7.60
SAC + FW	55.00 ± 14.80	92.38 ± 31.35	6.45 ± 1.57	38.50 ± 8.04
SF + PDW	65.50 ± 14.96	86.75 ± 23.21	7.64 ± 0.66	37.25 ± 4.16
SF + FW	63.83 ± 17.20	77.50 ± 16.07	6.32 ± 0.70	36.50 ± 3.67

Plasma activity of ALT and ALP and plasma concentration of BUN and creatinine were similar across treatment regimens. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; ALP = alanine phosphatase; ALT = alanine aminotransferase; BUN = blood urea nitrogen. Data presented as mean ± SD; n = 8-9 per treatment.

The treatment regimens had no significant effect on the plasma activity of alanine aminotransferase and alanine phosphatase and plasma concentrations of creatinine and blood urea nitrogen of the male rats (Table 4.17).

Table 4.18 shows the plasma activities of alanine aminotransferase and alanine phosphatase and plasma concentrations creatinine and blood urea nitrogen of the female rats following their respective treatment regimens.

Table 4.18: Effects of high-fructose diet on surrogate markers of liver and kidney function of adult male rats orally administered S-allyl-cysteine during suckling

Treatments	ALT (U/L)	ALP (U/L)	BUN (mg/dL)	Creatinine (mg/dL)
DH + PDW	53.17 ± 13.23	56.83 ± 7.68	6.25 ± 0.65	39.50 ± 4.93
DH + FW	54.25 ± 12.20	55.25 ± 19.65	6.088 ± 1.14	39.63 ± 8.10
FS + PDW	54.13 ± 7.04	56.75 ± 14.72	7.40 ± 1.08	46.25 ± 9.31
FS + FW	49.38 ± 8.23	56.38 ± 13.56	6.39 ± 1.45	40.63 ± 6.70
SAC + PDW	49.78 ± 19.80	68.78 ± 9.13	7.425±1.313	41.88 ± 11.34
SAC + FW	53.00 ± 9.70	62.00 ± 10.18	6.014 ± 0.87	39.00 ± 14.32
SF + PDW	52.71 ± 6.07	74.29 ± 15.20	6.94 ± 1.17	45.29 ± 9.62
SF + FW	56.11 ± 14.95	55.11 ± 12.60	6.88 ± 1.26	42.00 ± 3.97

Plasma activity of ALT and ALP and plasma concentration of BUN and creatinine were similar across treatment regimens. **DH + PDW** = gavage with 10 ml/kg body mass per day distilled water during suckling + plain drinking water in adulthood; **DH + FW** = gavage with 10 ml/kg body mass per day distilled water during suckling + 20% fructose (w/v) in their drinking water in adulthood; **FS + PDW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **FS + FW** = gavage with 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SAC + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + plain drinking water in adulthood; **SAC + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine during suckling + 20% fructose (w/v) in their drinking water in adulthood; **SF + PDW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + plain drinking water in adulthood; **SF + FW** = gavage with 150 mg/kg body mass per day S-allyl-cysteine and 10 ml/kg body mass per day 20% fructose solution (w/v) during suckling + 20% fructose (w/v) in their drinking water in adulthood; ALP = alanine phosphatase; ALT = alanine aminotransferase; BUN = blood urea nitrogen. Data presented as mean ± SD; n = 7-9 per treatment.

The treatment regimens had no significant effect on the plasma activity of alanine aminotransferase and alanine phosphatase and plasma concentrations of creatinine and blood urea nitrogen of the female rats (Table 4.18).

4.3 Discussion

The consumption of high-fructose diets is associated with the development of metabolic derangements inclusive of visceral obesity, insulin resistance and atherogenic dyslipidaemia. This current study aimed to investigate whether neonatal orally administered SAC protects Wistar rats against adverse metabolic outcomes induced by early or late ‘single-hit’ or ‘double-hit’ high-fructose insult. We found that ‘early single-hit’ neonatal orally administered 20% fructose solution programmed increased liver lipid accretion and microvesicular steatosis in adult male and female rats. However, exposure to the ‘double-hit’ (early and late) high-fructose did not result in exacerbated hepatic lipid accumulation in adulthood. Consumption of a ‘late single-hit’ high-fructose diet in adulthood resulted in visceral obesity. Neonatal orally administered SAC protected against programming of increased liver lipid accretion by neonatal orally administered 20% fructose solution but did not protect rats against visceral obesity induced by high-fructose diet consumption in adulthood. In the current study, the treatment regimens had no effect on growth performance, glucose tolerance, blood parameters, visceral organs morphometry, and kidney and liver function of male and female rats.

4.3.1 Growth performance

The current study reports no significant differences in the body masses of the male and female rats across treatment regimens at weaning and termination. However, both male and female rats across treatment regimens showed significant growth from weaning to termination. This suggests that the treatment regimens had no adverse effect on growth (body mass) of the rats. Work by Huynh *et al.* (2008) documented increased adulthood body masses and increased risk for obesity in Wistar rats to which a 10% fructose solution (first-hit) was orally administered during suckling and then fed 65% high-fructose diet (second-hit) in adulthood. The results of the current study where the neonatal oral administration of a 20% fructose solution represents the first-hit and the consumption of a 20% fructose drinking water in adulthood represents a second-hit, are at variance with the observations by Huynh *et al.* (2008). We speculate that the observed higher body masses and increased risk of obesity as reported by Huynh *et al.* (2008) could have been due to the higher caloric intake as 65% of the diet (in adulthood) was made up of fructose.

As discussed earlier, body mass is influenced by various factors in the short term, a longer term assessment of growth of growing animals is an assessment of various indices of the long bones. The similarity in the tibiae lengths, masses and Seedor indices of male and female rats across treatment regimens suggests that all the treatment regimens, including neonatal orally administered SAC, did not compromise or accelerate the growth performance of the rats. Previous studies have shown that the extracts and phytochemicals (diallyl sulphides and γ -L-glutamyl-trans-s-1-propenyl-L-cysteine-sulfoxide) of the *Amaryllidaceae* family plants, namely garlic, onion and leeks have been shown to increase bone mass and density by increasing calcium absorption in the GIT and inhibiting osteoclasts and bone resorption in animals (Ebrahimzadeh-Bideskan *et al.*, 2015; Ehnert *et al.*, 2012; Chen *et al.*, 2011; Mukherjee *et al.*, 2006; Mühlbauer *et al.*, 2002). Even though SAC is a phytochemical constituent of garlic, it can be orally administered in neonatal life as a prophylactic or therapeutic agent without adversely affecting growth performance.

4.3.2 Glucose tolerance, blood parameters, and liver lipid

While the oral glucose tolerance test (OGTT), a screening test for diabetes mellitus and pre-diabetes mellitus (Hage *et al.*, 2013), was established to evaluate the capability to handle an oral glucose load over a period time (Stumvoll *et al.*, 2000), the total area under the curve (AUC) for OGTT is computed to evaluate the total increase in blood glucose over a period of time following an oral glucose load (Sakaguchi *et al.*, 2012; Le Floch *et al.*, 1990). An increased AUC for OGTT is indicative of a decreased ability to effectively respond to an oral glucose load (Sakaguchi *et al.*, 2012). However, OGTT and AUC of OGTT are not adequate for evaluating insulin resistance. Thus the, minimally invasive, homeostatic model assessment of whole body insulin resistance (HOMA-IR) is used to quantify and evaluate insulin resistance (Matthews *et al.*, 1985). An increased HOMA-IR is indicative of insulin resistance (Matthews *et al.*, 1985).

The current study reports no differences in glucose tolerance, the area under the curve for the oral glucose tolerance test, insulin concentration and insulin resistance (HOMA-IR) of male and female rats across treatment regimens. These findings suggest that treatment regimens did not affect the ability to tolerate an oral glucose load, insulin secretion neither did they cause insulin resistance. Studies have shown that the consumption of 20% fructose drinking

water (w/v) for 8 weeks causes hyperglycaemia, hyperinsulinemia, insulin resistance, hypertriglyceridemia and hypercholesterolemia in adult rats (Sandeve *et al.*, 2015; Mamikutty *et al.*, 2014; Benado *et al.*, 2004; Dai and McNeill, 1995). Our findings suggest that the consumption of ‘late single-hit’ 20% fructose drinking water (w/v) for 8 weeks did not result in hyperglycaemia, hyperinsulinemia, insulin resistance, hypertriglyceridemia and hypercholesterolemia in male and female adult rats.

In the current study, the ‘double-hit’ (early and late) fructose had no effect on fasting blood glucose concentration, plasma triglyceride, cholesterol and insulin concentration and insulin resistance of adult male and female rats. The findings from the current study on fasting blood glucose concentration are in accordance with Huynh *et al.* (2008) who reported no changes in the blood glucose concentration of Wistar rats administered a 10% fructose solution (first-hit) during suckling and fed a 65% high-fructose diet (second-hit) in adulthood. Huynh *et al.* (2008), whose findings are at variance with those of the current study, reported hyperinsulinemia and insulin resistance in Wistar rats that were orally administered a 10% fructose solution (first-hit) during suckling and fed 65% high-fructose diet (second-hit) in adulthood. Similarly, Sánchez-Lozada *et al.* (2007) reported rats fed a diet with 60% fructose in feed (w/w) for 8 weeks developed more severe metabolic derangements than rats that consumed of 10% fructose solution and standard rat chow for 8 weeks. The differences observed by Sánchez-Lozada *et al.* (2007) were attributed to the 60% fructose in feed (w/w) providing surplus calories as compared to the 10% fructose solution (w/v). Thus the variance in our finding and those by Huynh *et al.* (2008) may have been due to the variation in the method by which the ‘second-hit’ high-fructose diet was administered in adulthood [20% fructose drinking water (w/v) used in the current study versus 60% fructose in feed used by Huynh *et al.* (2008)].

Fructose metabolism in the liver produces substrates for *de novo* lipogenesis (Wagnerberger *et al.*, 2013). This results in decreased β -oxidation of fatty acids and increased hepatic lipid accumulation (Nomura and Yamanouchi, 2012) and subsequently dyslipidaemia and NAFLD (Liu *et al.*, 2014). The similarity in the plasma triglyceride and cholesterol concentration of the rats across treatment regimens suggests that the treatment regimens did not alter plasma lipid (triglycerides and cholesterol) concentrations. Ching and colleagues (2011) reported the consumption of a high-fructose diet caused hypercholesterolemia, hypertriglyceridemia and increased hepatic lipid in Sprague-Dawley rats that were born to dams that were fed on a

high-fructose diet during gestation and lactation. Maternal high-fructose diet consumption during gestation and lactation has been shown to programme in resulting offspring, increased expression of lipogenic genes and increased susceptibility to developing hypertriglyceridemia and hypercholesterolemia (Ching *et al.*, 2011; Alzamendi *et al.*, 2010).

Findings from the present study suggest that unlike the case with maternal high-fructose diet consumption where the rat pups are exposed to high-fructose during gestation and while suckling, despite the altricial developmental state of rat pups, neonatal orally administered 20% fructose solution did not programme for increased susceptibility to develop hypertriglyceridemia and hypercholesterolemia in adult rats. The dissimilarities in our findings and those reported by Ching *et al.* (2011) and Alzamendi *et al.* (2010) could be related to variation in the duration of dietary (high-fructose diet) intervention and the model (neonatal oral administration of 20% fructose solution (current study) versus maternal high-fructose diet consumption (Ching *et al.* (2011) and Alzamendi *et al.* (2010)). The maternal high-fructose diet from gestation to lactation model used by Ching *et al.* (2011) and Alzamendi *et al.* (2010) caused hypertriglyceridemia and hypercholesterolemia in the rat dams however it is unclear whether the increased plasma lipids were directly transferred to the pups via the placenta or indirectly through milk during suckling. The increased plasma lipids in the dams may also alter how the foetuses develop in perinatal life (gestation and lactation) (Rodríguez *et al.*, 2013). It is known that rat dams fed high-fructose diet produce milk that is rich in fatty acids thus contributing to hyperlipidaemia in the suckling pups (Mourot *et al.*, 2010). In humans, fructose crosses the placenta (Goran *et al.*, 2013), although we were unable to find literature on whether the same applies to rats. Humans and rodents have a haemochorial type of placentation (Soares *et al.*, 2012) thus one would expect that there would be similarities in the maternal-foetal transfer of substances. However, Vickers *et al.* (2011) reported that despite increased plasma fructose concentration in high-fructose diet fed dams there was no increase in fructose in the milk that was extracted from the stomach of suckling pups. It is thus unlikely that the fructose is directly transferred through the milk during suckling. Thus the differences in the findings on plasma lipids of rats from the current study, which used a high-fructose neonatal diet model, and those reported by Ching *et al.* (2011), who used a high-fructose maternal diet model could be attributed to the variance in the route of fructose exposure [direct neonatal fructose exposure used by the current study versus indirect foetal/neonatal fructose exposure used by Ching *et al.* (2011)].

In the current study, we showed that the accumulation of hepatic lipids was affected not only by the developmental age when the fructose was administered but also by the sex of the rats. Neonatal oral administration of a 20% fructose solution ‘early single-hit’ significantly increased liver lipid accretion in male and female adult rats (Tables 4.11 and 4.12 respectively) despite them not receiving any more excessive fructose later in adult life. Our findings suggest that neonatal oral administration of a 20% fructose solution single-handedly programmed for increased liver lipid accumulation in adulthood in male and female rats. The ‘early single-hit’ also resulted in the greatest accumulation of hepatic lipids in both sexes. Prenatal and or early postnatal maternal fructose consumption has been shown to alter expression of genes that are involved in lipogenesis and β -oxidation of free fatty acids (Yamazaki *et al.*, 2016; Clayton *et al.*, 2015; Rodríguez *et al.*, 2013). Huynh *et al.* (2008) also showed that neonatal orally administered 10% fructose solution alone programmed increased fatty acid uptake and deposition in the skeletal muscle of Wistar rats by increasing skeletal muscle fatty acid transporters. These mechanisms may explain our findings.

It was interesting that we found that the oral consumption of a high-fructose diet ‘late single-hit’ in adulthood increased liver lipid accretion in female rats only (Table 4.12). The current study also reports that exposure to a ‘double-hit’ high-fructose diet (early and late) increased liver lipid accretion in the adult male rats only (Table 4.12). However, the ‘double-hit’ (early and late) high-fructose diet did not cause a higher hepatic lipid accretion than did the ‘early single-hit’ high-fructose diet.

Thus, in summary, these findings suggest that both male and female rats were prone to developing fatty-liver disease when they were exposed to a high-fructose diet (20% fructose solution) as neonates, however the male rats were more prone than females to developing fatty-liver disease when they were exposed to ‘double-hit’ (early and late) high-fructose diet. The similarities in the liver lipid content of female rats subjected to a ‘double-hit’ of fructose and their counterparts on the control treatment regimen (Table 4.12), suggests that the ‘double-hit’ high-fructose diet does not exacerbate neonatal fructose-induced programming of increased liver lipid accumulation. We hypothesise that this may be related to the anti-insulinotropic and lipid-lowering effects of the high-fructose diet that were reported in the first experiment of the current study. These anti-insulinotropic and lipid-lowering effects of fructose in neonatal life may have imparted some adaptive effect to a high fructose diet in

later life. There is a need to interrogate at the cellular and molecular level the possible mechanisms that could explain the findings of the current study.

An exciting finding in the current study was the observation that the neonatal oral administration of a combination of SAC + 20% FS to neonates (early fructose single-hit) without further excess fructose in adulthood resulted in significantly reduced liver lipid in the male rats (Table 4.11). As discussed earlier the ‘early single-hit’ of fructose resulted in the greatest accumulation of liver lipids in both males and females. However, neonatal oral administration of a combination of SAC and 20% fructose solution followed by 20% fructose drinking water in adulthood (double-hit with fructose) significantly increased liver lipid content in the male rats (Table 4.11). These findings suggest that even though neonatal orally administered SAC protected male rats against the programming of increased liver lipid accretion induced by neonatal orally administered 20% fructose solution (single early fructose hit), it (neonatal oral SAC administration) did not protect the male rats against increased liver lipid accumulation induced by ‘double-hit’ (neonatal and adult) fructose insult.

The current study reports similarities in the liver lipid content from female rats on the control treatment regimen to that from female rats subjected to a combination of neonatal oral SAC and 20% fructose solution administration and or neonatal oral SAC administration with a high-fructose diet (20% fructose drinking water) in adulthood (Table 4.12). These findings suggest that neonatal oral administration of SAC protected female rats against increased liver lipid accretion induced by the adverse programming effects of neonatal orally administered 20% fructose solution or high-fructose diet administered in adulthood. The protective effects of SAC are probably related to its antidiabetic, antiobesity and antioxidant activities as reported by several researchers (Asdaq, 2015; Saravanan *et al.*, 2013; Saravanan and Pomurugan, 2012a).

Interestingly, in the current study both male and female rats to which SAC was orally administered during their neonatal growth stage and received plain drinking water during adulthood had significantly higher liver lipid content compared to their counterparts on other treatment regimens (Tables 4.11 and 4.12, respectively). This finding suggests that similar to the neonatal orally administered 20% fructose solution, the neonatal orally administered SAC programmed rats for increased liver lipid accumulation in adulthood. In the first experiment (chapter 3) we reported that the beneficial (insulinotropic) effects neonatal orally

administered SAC may have caused the increased liver lipid accumulation that was observed in the suckling female rat pups. We, therefore, suspect that these observations may be linked to the insulinotropic effects of neonatal orally administered SAC that we noted in the first experiment (neonatal study).

We propose that the sexually dimorphic effects of treatment regimens on the liver lipid accretion that were noted in the current study (discussed above) could be attributed to the variance in the sex hormones and physiological development in early-life between male and female rats. While testosterone is the principal sex hormone in males, in females the main sex hormones are oestrogen and progesterone (Juster *et al.*, 2016; Kadowaki *et al.*, 2006). Although the mechanisms are not yet fully understood, studies have shown that testosterone and oestrogen are involved in the regulation of various metabolic pathways (Kadowaki *et al.*, 2006) and thus play a role in the onset and prevalence various metabolic derangements such as NAFLD and MetS (Kim *et al.*, 2012; Carulli *et al.*, 2006). In male rats, the neural and endocrine systems have been shown to mature at an earlier age than in female rats (Sakuma, 2009; Viveros *et al.*, 2009) and thus could have contributed to sexually dimorphic responses to the treatment regimens. Even though our proposed theories could potentially explain the sexually dimorphic effects of the treatment regimens on liver lipid accretion (discussed earlier), there still is a need to further interrogate and identify the physiological mechanisms which attributed the sexually dimorphic responses or effects noted in the current study.

4.3.3 Visceral organs morphometry

Orally administered raw garlic juice was reported to cause injury to the intestinal lining resulting in intestinal atrophy in adult rats (Amagase *et al.*, 2001). The observed adverse effect of garlic juice could be attributed to it containing multiple phytochemicals including allicin which has been reported to cause oxidative damage to the intestinal mucosa (Lawson, 1993). The similarities in macro-morphometric parameters (masses and lengths) of the liver, small and large intestines of male and female rats across treatment regimens suggest that the treatment regimens did not compromise nor promote growth and development of viscera. Our findings suggest that neonatal orally administered SAC, which is a phytochemical constituent of garlic, did not cause atrophy or hypertrophy of visceral organs. Thus SAC can be orally

administered in neonatal life as a prophylactic agent without compromising the growth and development of the liver and GIT viscera.

While increased visceral fat accumulation is associated with insulin resistance and the development of MetS, DM II and cardiovascular diseases (Esser *et al.*, 2014; DeBoer, 2013; Makki *et al.*, 2013), epididymal fat accumulation has been linked to infertility in males (Katib, 2015). In the present study, the ‘late single-hit’ or ‘double-hit’ (early and late) high-fructose diet caused significantly heavier visceral and epididymal (in male rats) fat masses in male and female rats (Tables 4.9 and 4.10). Oral administration of ‘early single-hit’ high-fructose diet had no effect on visceral and epididymal (in male rats) fat pad masses of male and female rats in adulthood (Tables 4.9 and 4.10). Our findings suggest that while the consumption of a ‘late single-hit’ high-fructose diet caused increased visceral and epididymal (in male rats) fat accumulation in rats, the orally administered ‘early single-hit’ high-fructose diet did not predispose the male and female rats to have increased visceral and epididymal (in male rats) fat accumulation in adulthood. The similarity in the visceral and epididymal (in male rats) fat pad masses of adult rats that were exposed to ‘late single-hit’ or ‘double-hit’ (early and late) high-fructose diet suggests that although the ‘double-hit’ high-fructose diet caused increased visceral and epididymal (in male rats) fat accumulation, it did not exacerbate the adiposity. Importantly our findings suggest that the ‘late single-hit’ or ‘double-hit’ (early and late) high-fructose diet caused visceral obesity in the adult rats and thus increasing their susceptibility to developing metabolic derangements such as insulin resistance, MetS, and DM II. Additionally, exposure to the ‘late single-hit’ or ‘double-hit’ high-fructose diet increased epididymal fat accumulation in male rats and thus potentially increasing their risk of infertility (El-Wakf *et al.*, 2015; Katib, 2015). Our findings are in agreement with several works that have indicated that high-fructose diet is associated with visceral obesity and increased epididymal fat pad accumulation (Pektaş *et al.*, 2015; Tran *et al.*, 2009; Huynh *et al.*, 2008).

In the current study, neonatal orally administered SAC did not protect the rats against visceral and epididymal (in male rats) fat accumulation induced by a ‘late single-hit’ and or ‘double-hit’ high-fructose diet. These findings suggest that even though SAC showed protective effects against the high-fructose diet-induced excessive hepatic lipid accretion, it did not protect the rats against increased visceral and epididymal fat (male) accumulation induced by the ‘late single-hit’ and ‘double-hit’ high-fructose diet.

Non-alcoholic fatty liver disease is a progressive disease, and thus fatty liver disease scoring criteria systems such as the NAFLD activity score (NAS) are pivotal for evaluating and predicting the progression and severity of NAFLD (Kleiner *et al.*, 2005). The NAS uses a semi-quantitative scoring system using the following histological criteria: steatosis, lobular inflammation and ballooning (Kleiner *et al.*, 2005). Steatosis refers to the excessive accumulation of lipid in the liver and can be categorised as macrovesicular and microvesicular steatosis (Nativ *et al.*, 2014). Whilst the former (macrovesicular) steatosis, is characterised by few large fat droplets within the hepatocytes and displaced nuclei (Mulder *et al.*, 2015), the latter (microvesicular) steatosis, is characterised by multiple tiny lipid droplets within the hepatocytes (Mulder *et al.*, 2015). The manifestation of steatosis with lobular inflammation and ballooning is indicative of fatty-liver disease that has progressed to steatohepatitis with hepatocyte degeneration (Kleiner *et al.*, 2005). While lobular inflammation characterised by mononuclear cell infiltration of the hepatic lobule, ballooning is characterised by hepatocyte apoptosis, cytoplasm clearing and enlargement (Kleiner *et al.*, 2005).

The current study reports the presence of microvesicular steatosis in the liver of male and female rats that were orally administered an ‘early single-hit’ fructose diet (20% fructose solution) only or ‘late single-hit’ fructose (20% fructose drinking water) only (Figures 4.11 and 4.12). These findings suggest that ‘single-hit’ (neonatal or adulthood) and ‘double-hit’ fructose-diet exposure caused microvesicular steatosis in male and female rats and support our findings which suggested that neonatal oral administration of fructose programmed for increased liver lipid accretion in adulthood. Microvesicular steatosis was not observed in the liver histology sections of male and female rats that were orally administered SAC and 20% fructose solution during suckling and received plain drinking water in adulthood. This further suggests that neonatal orally administered SAC attenuated the programming of increased liver lipid accretion induced by neonatal oral administration of a 20% fructose solution. The present study also reports the presence of microvesicular steatosis in the liver of male rats that were orally administered of a combination of neonatal SAC and 20% fructose solution followed by the consumption of 20% fructose drinking water in adulthood (double-hit with fructose) and in female rats that were orally administered neonatal SAC only (Figures 4.11 and 4.12). These findings suggest that neonatal oral administration of SAC caused microvesicular steatosis in female rats and did not protect the male rats against microvesicular steatosis induced by ‘double-hit’ (early and late) high-fructose diet. The

microvesicular steatosis that was induced by neonatal orally administered SAC in adult female rats may be related to its (SAC's) programming of increased liver lipid accretion in adulthood. Microvesicular steatosis is associated with decreased hepatic β -oxidation and mitochondrial dysfunction (Tandra *et al.*, 2011). Thus the observed microvesicular steatosis may possibly signify symptoms of decreased hepatic β -oxidation and hepatic mitochondrial dysfunction.

Even though we observed increased liver lipid accretion and hepatic microvesicular steatosis in the rats that were exposed to early or late 'single-hit' or double-hit fructose insult (Tables 4.11 and 4.12; Figures 4.11 and 4.12), there were similarities in the number of hepatocytes in a linear field, hepatocyte size, hepatocyte ballooning score, steatosis score, inflammation score, and total NAFLD activity score (NAS) of male and female rat across treatment regimens. This suggests that despite increased liver lipid accretion and the presence of microvesicular hepatic steatosis in the rats that were exposed to early or late 'single-hit' or 'double-hit' high-fructose, none of the treatment regimens had adverse effects on hepatocyte density or caused NASH.

4.3.4 Surrogate markers of liver and kidney function

The similarity in surrogate markers of liver (ALT and ALP activity) and kidney (creatinine and blood urea nitrogen concentration) function of male and female rat across treatment regimens in the present study suggests that the treatment regimens no adverse effects on liver and kidney function. The consumption of high-fructose diets, mainly in feed, has been observed to cause renal and hepatic impairments that manifest in the form of decreased excretory function resulting in increased blood urea nitrogen and creatinine concentration (Johnson *et al.*, 2010; Sánchez-Lozada *et al.*, 2008; Johnson *et al.*, 2007) and hepatocellular damage that results in increased activity of ALT and aspartate aminotransferase (AST) (Hou *et al.*, 2014). None of the high-fructose diet (early single-hit, late single-hit and double-hit) interventions used in the current study resulted in renal or hepatic impairments. This could be attributed to the variance in the amount of fructose administered (20% fructose drinking water used in the current versus 60% fructose in feed used by Johnson *et al.* (2010), Sánchez-Lozada *et al.* (2008) and Johnson *et al.* (2007).

An important aspect of studies aimed validating the efficacy of phytochemicals as ethnomedicines is assessing their potential toxicity. In antiobesity studies, where weight loss is evaluated, an assessment of toxicity would eliminate the potential of pseudo-medicinal induced weight loss due to the toxicity of the ethnomedicinal phytochemicals under validation. Our findings show that neonatal orally administered SAC did not have any long-term toxic or adverse effects on the liver and kidney and thus can be administered during neonatal development phase as a prophylactic agent for high-fructose diet-induced fatty-liver disease.

4.4 Conclusion

There are similarities and differences in the responses of the sexes to high-fructose diets and neonatal SAC. The timing of high-fructose diet intervention also plays a role in the response. The consumption of a 'late hit' high-fructose diet in adulthood, regardless of early intervention, resulted in visceral obesity in both male and female rats. The increased visceral obesity was not attenuated in rats where SAC was orally administered during the neonatal growth stage. Neonatal oral administration of a 20% fructose solution (single-hit) programmed the male and female rats to develop increased liver lipid accretion in adulthood. Thus the suckling growth stage is an important period of plasticity where the oral administration of a 20% fructose solution (simulating a high-fructose diet) exerted adverse metabolic programming effects on the liver lipid accretion and the possibility of an increased risk of developing fatty liver disease(s) in adulthood. Importantly, the programming of increased liver lipid accretion by the 'single-hit' neonatal oral administration of a 20% fructose solution was mitigated by neonatal oral administration of SAC in male and female rats. We thus conclude that neonatal oral administration of SAC to suckling Wistar rat pups protected them against adverse fructose-induced neonatal metabolic programming of fatty livers in adulthood. We suspect that the mechanisms by which SAC exerted the beneficial/protective effects are mainly related to its insulinotropic effects. This inference requires further probing and validation. It should also be noted that although neonatal orally administered SAC showed beneficial effects in a sexually dimorphic manner, its (SAC) use has the potential to cause fatty-liver disease.

The next chapter outlines the major conclusions from the study, points out the limitations to the study and suggests recommendations for future studies.

**CHAPTER 5: CONCLUSION,
LIMITATIONS AND
RECOMMENDATIONS**

The current study was executed in two major experiments. In the first experiment we investigated the potential of neonatal orally administered SAC (150 mg/kg body mass per day) to protect against the development of high-fructose diet-induced metabolic derangements by specifically determining its effects on growth performance, blood parameters, liver metabolites, and surrogate markers of liver and kidney function of suckling Wistar rats to which a 20% fructose solution (high-fructose diet) was orally administered. There is increased plasticity during the suckling period and thus dietary interventions during this period can alter growth, development and function of various physiological systems. The findings of the first experiment were as follows:

- i. neonatal oral administration of 20% fructose solution induced hypoinsulinemia in female rat pups but not in their male counterparts suggesting that female rat pups were susceptible to the insulin-lowering effects of neonatal orally administered 20% fructose solution. This is one of the first animal studies to show that neonatal oral administration of 20% fructose solution decreases plasma insulin in female rat pups. The mechanisms by which the neonatal orally administered 20% fructose solution reduced plasma insulin concentration in female rat pups are unclear. We theorise that mechanisms could be related to the delayed development of the endocrine system in female rats and possibly to fructose altering satiety via the hypothalamus-pituitary-adrenal-leptin axis. However, leptin and ghrelin which are involved in the regulation of satiety and pancreatic β -cell development and functionality were not determined in the current study. We thus recommend that future studies evaluate leptin, ghrelin and pancreatic β -cell development and functionality when investigating the effects of high-fructose diets in early-life (gestation and lactation). This might provide more insight into the possible mechanisms by which neonatal oral administration of 20% fructose solution decreases blood insulin concentration in female rats.
- ii. neonatal orally administered SAC attenuated the high-fructose diet-induced hypoinsulinemia in female rat pups, indicating potential insulinotropic effects of SAC in female pups.
- iii. neonatal orally administered SAC increased insulin in male pups. These findings suggest that even though SAC exerted insulinotropic effects in both male and female rat pups, these effects were more pronounced in male pups. Thus SAC potentially could be used to ameliorate insulin related disturbances in diabetics. Future studies need to further investigate the insulinotropic effects of SAC, potential adverse outcomes from

SAC usage and potential exploitation of SAC as a prophylactic agent for the prevention, management, and treatment of metabolic disturbances such as DM II.

- iv. neonatal orally administered SAC increased liver lipid accretion in female rat pups. This suggests that the use of SAC in young females might increase the risk of developing NAFLD. The current study did not evaluate the lipid profile of the hepatic lipids. This would have provided insight on the types of lipids and fatty acids that accumulated in the liver due to the neonatal oral administration of SAC in suckling female rat pups. Thus we recommend that future studies should consider evaluating the lipid profile when analysing tissue lipid content. Additionally the activity and expression of hepatic lipogenic enzymes, proteins and genes would possibly provide some clarifications on the possible mechanisms by which SAC increased hepatic liver lipid content in female rat pups.

In the second experiment, we investigated whether neonatal oral administration of SAC conferred protection against an ‘early or late single-hit’ or ‘double-hit’ fructose-induced metabolic derangement in adulthood. The findings of the second experiment were as follows:

- i. the consumption of a high-fructose diet in adulthood increased visceral fat of male and female rats. However, neonatal oral administration of SAC did not protect rats against visceral obesity induced by high-fructose diet consumption in adulthood.
- ii. ‘single-hit’ neonatal orally administered fructose programmed increased liver lipid accretion without causing overt NAFLD in adulthood in male and female rats, indicating that male and female rats were susceptible to the programming of increased liver lipid accumulation in adulthood induced by neonatal oral administration of ‘single-hit’ 20% fructose solution. ‘Early single-hit’ high-fructose diet exerted programming effects mainly in the liver and may have increased the risk of developing NAFLD. It should be noted that in the neonatal (first experiment) study changes in liver lipid and glycogen content were noted in suckling female rats orally administered 20% fructose solution only. This suggests that while female rats are susceptible to the short- and long-term adverse effects of neonatal orally administered 20% fructose solution, the male rats are resistant to the short-term adverse effects of neonatal orally administered 20%

fructose solution and are susceptible to the long-term adverse effects of neonatal orally administered 20% fructose solution.

- iii. a 'late single-hit' high-fructose diet increased liver lipid accretion in female rats but not in male rats and 'double-hit' (early and late) high-fructose diet increased liver lipid accretion in male rats but not in female rats. These findings suggest that while female rats are susceptible to the increased liver lipid induced by 'single-hit' high-fructose diet consumption in adulthood the male rats are susceptible to increased liver lipid induced by 'double-hit' fructose.
- iv. neonatal oral administration of SAC attenuated the programming of increased liver lipid accretion induced by neonatal oral administration of 'single-hit' 20% fructose solution during suckling in male and female rats. Firstly, these findings suggest that neonatal oral administration of SAC attenuated increased liver lipid accretion induced by neonatal oral administration of 'single-hit' 20% fructose solution in adult female rats. Secondly, neonatal oral administration of SAC protected male and female rats against programming of liver lipid accumulation induced by neonatal oral administration of 'single-hit' 20% fructose solution. Neonatal oral administration of SAC protected female rats against increased liver lipid accretion induced by 'single-hit' high-fructose diet consumption in adulthood but it did not protect male rats against increased liver lipid induced by 'double-hit' fructose. We thus conclude that neonatal orally administered SAC protected against the adverse programming of increased liver lipid accretion by neonatal 'single-hit' with a 20% fructose solution in both male and female rats. However, SAC may exert some of its protective effects in a sexually dimorphic manner. The mechanisms by which SAC exerted the beneficial/protective effects against the adverse effects of the 20% fructose solution (at the neonatal stage) and a high-fructose diet (adulthood) model utilised by the current study have not been elucidated. However, we theorise that the mechanisms may be related to the antiobesity, antidiabetic, antioxidant and hepatoprotective activities of SAC, which were reported by other studies (Asdaq, 2015; Takemura *et al.*, 2013; Saravanan *et al.*, 2010).
- v. neonatal oral administration of SAC alone programmed increased liver lipid accretion without causing NAFLD in male and female rats, indicating that neonatal oral administration of SAC exerted programming effects which resulted in increased non-pathological liver lipid accretion in both male and female rats in adulthood. Importantly, in the neonatal (first stage experiment) study, neonatal orally administered SAC increased liver lipid in suckling female rat pups and increased insulin in male rat pups.

The programming effects of SAC could be linked to the metabolic changes exerted by neonatal oral administration of SAC. Nonetheless, the mechanisms by which neonatal orally administered SAC programmed increased liver lipid accretion in adulthood in male and female rats still require interrogation. To shed some light on the possible mechanisms, future studies should consider including investigations on the molecular components such as the effects of SAC on the methylation, modification and regulation genes that code for lipogenic enzymes.

Overall we conclude that the period of suckling is a critical period where dietary interventions such as high-fructose diet can exert short- and long- term adverse metabolic programming effects that increase the susceptibility to metabolic derangements in a sexually dimorphic manner. Thus we recommend that prophylactic use of antidiabetic and antiobesity interventions such as SAC during the suckling period could aid in mitigating the incidence of obesity, NAFLD, MetS and DM II in childhood and adulthood. We conclude that the neonatal orally administered SAC has the potential to protect specific tissues against the short- and long-term adverse outcomes of fructose-induced metabolic programming. However, we acknowledge the need to validate the mechanisms involved in the beneficial outcomes of neonatal orally administered SAC. Future studies should always use both male and female rats when investigating the metabolic programming so that they do not omit on the sexually dimorphic responses. Moreover, such studies should perform an assessment on multiple physiological systems and organs. This will ensure that physiological system specific and tissue specific responses are not omitted.

CHAPTER 6: REFERENCES

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APPENDICES

Appendix 1: Animal ethics clearance certificate



STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2015/07/B

APPLICANT: Mr BW Lembede

SCHOOL: Physiology

DEPARTMENT:

LOCATION: Medical School

PROJECT TITLE: Effects of allicin on high fructose diet induced neonatal programming in Wistar rats

Number and Species

320 Wistar rat pups and 32 nursing Wistar rat dams

Approval was given for to the use of animals for the project described above at an AESC meeting held on 2015/02/24. This approval remains valid until 2017/02/23.

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:

Signed: _____

(Chairperson, AESC)

Date: _____

13th March 2015

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: _____

12th March 2015

cc: Supervisor: Dr E Chivandi
Director: CAS

Works 2000/1ain0015/AESCCert.wps

Appendix 2: Modification of the ethics clearance

AESC 2015 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND ANIMAL ETHICS SCREENING COMMITTEE MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

- a. Name: Busisani Lembede
b. Department: School of Physiology

c. Experiment to be modified / extended

AESC NO

Original AESC number	2015	07	B
Other M&Es :			

- d. Project Title: Effects of allicin on high fructose diet induced neonatal programming in Wistar rats

	No.	Species
e. Number and species of animals originally approved:	320 32	<i>Wistar rat pups</i> <i>Nursing Dams</i>
f. Number of additional animals previously allocated on M&Es:	0	
g. Total number of animals allocated to the experiment to date:	0	
h. Number of animals used to date:	0	

i. Specific modification / extension requested:

1. I would like to add co-workers:
 - Dr Neville Pitts [BSc (Hons) MSc, PhD]; 011 7172461.
 - Jeanette Joubert (BSc); 011 7172413:
2. I would like to change the phytochemical allicin that I initially intended to use in the study, instead I would like to use the peptide form of the phytochemical allicin namely S-allyl-L-cysteine.

j. Motivation for modification / extension:

1. Dr Pitts will provide technical assistance with assays for antioxidants and hormones in blood collected during the study.

Jeanette Joubert is an honours student and will provide assistance with collection of data and animal husbandry. She will also use some of the data for their Honours project.

2. Further interrogation of the literature has revealed that allicin has decreased bioavailability and higher potential to cause cytotoxic effects compared to its peptide form, a compound namely S-allyl-L-cysteine (Kodera *et al.*, 2002). S-allyl-L-cysteine has been demonstrated to be a bioactive compound that gives garlic its medicinal properties (Amagase, 2006). I would thus like to use S-allyl-L-cysteine for my studies. It will be administered at a dose of 150mg.kg⁻¹ (Saravanan *et al.*, 2013). All other procedures will remain as approved by the

AESC 2015 M&E

AESC.

Reference(s):

Amagase, H. (2006). Clarifying the real bioactive constituents of garlic. *The Journal of nutrition*, 136, 716S-725S.

Kodera, Y., Suzuki, A., Imada, O., Kasuga, S., Sumioka, I., Kanezawa, A., Taru, N., and Ono, K. (2002). Physical, chemical, and biological properties of S-allylcysteine, an amino acid derived from garlic. *Journal of agricultural and food chemistry*, 50, 622-632.

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Date: 11 May 2015

Signature: 

RECOMMENDATIONS: Approved.

- i. Inclusion of Dr Neville Pitts and Miss Jeanette Joubert as co-workers. Jeanette should attend the CAS course for first time Users of the facility.
- ii. Use of S-allyl-L-cysteine instead of allicin.

Date: 12 May 2015

Signature: 

Chairman, AESC

Appendix 3: NAFLD Activity Score

Non-Alcoholic Fatty Liver Disease (NAFLD) Activity Score (NAS)-semi quantitative for 1, 2 and 3.

A. Criteria assessed: 1. hepatocellular ballooning (H and E)

2. Steatosis (H and E)

3. Inflammation (H and E)

Location of the changes: perivenular (zone 3), perisinusoidal (zone 2) and periportal (zone 1).

1). Steatosis can be classified as macrovesicular or microvesicular.

B. Semi quantitative: Grading or Scoring

Scored from 0-3

Steatosis

0- < 5%

1- 5-33%

2- 33-66%

3- > 66%

Inflammation

0- None or no foci per camera field (approx. X200)

1- < 2 foci per camera field

2- 2-4 foci per camera field

3- > 4 foci per camera field

Ballooning

0. None

1. A few cells

2. Many cells and often prominent ballooning

C. Correlation between total NAFLD activity scores and an overall histological diagnosis of steatohepatitis

NAFLD activity score

>5

3-4

< 2

Histological diagnosis of steatohepatitis

Probable or definite NASH

Uncertain

Not NASH

Appendix 4: Rat insulin enzyme-linked immunosorbent assay (ELISA) kit protocol

6th Edition, revised in June, 2015

Assay procedure

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. **All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming.** It's recommended that all samples and standards be assayed in duplicate.

1. **Add Sample:** Add 100 μ L of Standard, Blank, or Sample per well. The blank well is added with Reference Standard & Sample diluent. Solutions are added to the bottom of micro ELISA plate well, avoid inside wall touching and foaming as possible. Mix it gently. Cover the plate with sealer we provided. Incubate for 90 minutes at 37°C.
2. **Biotinylated Detection Ab:** Remove the liquid of each well, don't wash. Immediately add 100 μ L of Biotinylated Detection Ab working solution to each well. Cover with the Plate sealer. Gently tap the plate to ensure thorough mixing. Incubate for 1 hour at 37°C.
3. **Wash:** Aspirate each well and wash, repeating the process three times. Wash by filling each well with Wash Buffer (approximately 350 μ L) (a squirt bottle, multi-channel pipette, manifold dispenser or automated washer are needed). Complete removal of liquid at each step is essential. After the last wash, remove remained Wash Buffer by aspirating or decanting. Invert the plate and pat it against thick clean absorbent paper.
4. **HRP Conjugate:** Add 100 μ L of HRP Conjugate working solution to each well. Cover with the Plate sealer. Incubate for 30 minutes at 37°C.
5. **Wash:** Repeat the wash process for five times as conducted in step 3.
6. **Substrate:** Add 90 μ L of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for about 15 minutes at 37°C. Protect the plate from light. The reaction time can be shortened or extended according to the actual color change, but not more than 30minutes. When apparent gradient appeared in standard wells, user should terminate the reaction.
7. **Stop:** Add 50 μ L of Stop Solution to each well. Then, the color turns to yellow immediately. The order to add stop solution should be the same as the substrate solution.
8. **OD Measurement:** Determine the optical density (OD value) of each well at once, using a micro-plate reader set to 450 nm. User should open the micro-plate reader in advance, preheat the instrument, and set the testing parameters.
9. After experiment, put all the unused reagents back into the refrigerator according to the specified storage temperature respectively until their expiry.

Important Note:

1. **ELISA Plate:** The just opened ELISA Plate may appear water-like substance, which is normal and will not have any impact on the experimental results.
2. **Add Sample:** The interval of sample adding between the first well and the last well should not be too long, otherwise will cause different pre-incubation time, which will significantly affect the experiment's accuracy and repeatability. For each step in the procedure, total dispensing time for

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addition of reagents or samples to the assay plate should not exceed 10 minutes. Parallel measurement is recommended.

3. **Incubation:** To prevent evaporation and ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Do not let the strips dry at any time during the assay. Strict compliance with the given incubation time and temperature.
4. **Washing:** The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings. Residual liquid in the reaction wells should be patted dry against absorbent paper in the washing process. But don't put absorbent paper into reaction wells directly. Note that clear the residual liquid and fingerprint in the bottom before measurement, so as not to affect the micro-titer plate reader.
5. **Reagent Preparation:** As the volume of Concentrated Biotinylated Detection Ab and Concentrated HRP Conjugate is very small, liquid may adhere to the tube wall or tube cap when being transported. You better hand-throw it or centrifugal it for 1 minute at 1000rpm. Please pipette the solution for 4-5 times before pipetting. Please carefully reconstitute Standards, working solutions of Detection Ab and HRP Conjugate according to the instructions. To minimize imprecision caused by pipetting, ensure that pipettors are calibrated. It is recommended to suck more than 10 μ L for once pipetting. Do not reuse standard solution, working solution of Detection Ab and HRP Conjugate, which have been diluted. If you need to use standard repeatedly, you can divide the standard into a small pack according to the amount of each assay, keep them at -20~-80 $^{\circ}$ C and avoid repeated freezing and thawing.
6. **Reaction Time Control:** Please control reaction time strictly following this product description!
7. **Substrate:** Substrate Solution is easily contaminated. Please protect it from light.
8. **Stop Solution:** As it is an acid solution, please pay attention to the protection of your eyes, hands, face and clothes when using this solution.
9. **Mixing:** You'd better use micro-oscillator at the lowest frequency, as sufficient and gentle mixing is particularly important to reaction result. If there is no micro-oscillator available, you can knock the ELISA plate frame gently with your finger before reaction.
10. **Security:** Please wear lab coats and latex gloves for protection. Especially detecting samples of blood or other body fluid, please perform following the national security columns of biological laboratories.
11. Do not use components from different batches of kit (washing buffer and stop solution can be an exception).
12. To avoid cross-contamination, change pipette tips between adding of each standard level, between sample adding, and between reagent adding. Also, use separate reservoirs for each reagent. **Otherwise, the results will be inaccurate!**