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THE POTENTIAL OF ZINGERONE ADMINISTERED ORALLY TO NEONATAL RATS AS PROTECTION AGAINST HIGH-FRUCTOSE DIET-INDUCED METABOLIC DERANGEMENTS



Nasiru Muhammad

Original published work submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2021.

DECLARATION

I, **Nasiru Muhammad**, student number **1332380**, declare that this thesis is my own work except otherwise acknowledged, and that I contributed adequately towards research findings published in the articles stated below which are included in my thesis. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg, Republic of South Africa. It has not been submitted before for any degree or examination at any other University. All the experimental procedures used in this thesis were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC number: 2017/010/71/B).

Signature of Student …… ..... **Date**……..02/06/2021.....

Name of Primary Supervisor……Professor Kennedy H. Erlwanger……..…..

Signature of Primary Supervisor … …**Date**……02/06/2021....

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the articles by the student as part of his thesis.

Article 1: Title: Neonatal zingerone protects against the development of high-fructose diet-induced metabolic syndrome in adult Sprague-Dawley rats.

Journal of Developmental Origins of Health and Disease, 2020, pp. 1-9,

doi: 10.1017/S2040174420000525

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2nd author	Dr. Busisani W. Lembede		02/06/2021
3rd author	Prof. Kennedy H. Erlwanger		02/06/2021

Comments by the primary supervisor:

Nasiru Muhammad as the first author of the manuscript was responsible for data collection, data analysis and writing up the first draft of the manuscript. He was also responsible for making

amendments following feedback from the reviewers. All of the work was done under our supervision and guidance. Where technical assistance was provided by others, it is duly acknowledged in the manuscript.

Article 2: Title: Zingerone administered neonatally prevents the subsequent development of high dietary fructose-induced fatty liver in Sprague Dawley rats.

Journal of Medicinal Food, 2021, pp 1-9. doi: 10.1089/jmf.2020.0189

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3rd author	Prof. Kennedy H. Erlwanger		02/06/2021

Comments by the primary supervisor:

Nasiru Muhammad as the first author of the manuscript was responsible for data collection, data analysis and writing up the first draft of the manuscript. He was also responsible for making amendments following feedback from the reviewers. All of the work was done under our supervision and guidance. Where technical assistance was provided by others, it is duly acknowledged in the manuscript.

Article 3: Title: Neonatal administration of zingerone prevents the subsequent development of high dietary fructose-induced early features of nephropathy in rats.

Submitted to the Journal of Animal Physiology and Animal Nutrition; 2021, and is currently under review (Manuscript ID: JAPAN-Apr-21-285).

Authors	Name	Signature	Date
1st author	Nasiru Muhammad		02/06/2021
2nd author	Dr. Busisani W. Lembede		02/06/2021
3rd author	Prof. Kennedy H. Erlwanger		02/06/2021

Comments by the primary supervisor:

Nasiru Muhammad as the first author of the manuscript was responsible for data collection, data analysis and writing up the first draft of the manuscript. He was also responsible for making amendments following feedback from the reviewers. All of the work was done under our supervision and guidance. Where technical assistance was provided by others, it is duly acknowledged in the manuscript.

DEDICATION

This work is dedicated with

love and affection to

my entire family

PRESENTATIONS ARISING FROM THIS RESEARCH PROJECT

Oral Presentation

1. **N. Muhammad,** B. W. Lembede and K. H. Erlwanger. "Neonatally administered zingerone prevents the development of metabolic dysfunction in high-fructose diet-fed female Sprague-Dawley rats". 47th Conference of the Physiology Society of Southern Africa (PSSA 2019), held at the International Conference Centre, East London, Republic of South Africa (18th- 21st August 2019).

Poster presentations

1. **N. Muhammad,** B. W. Lembede and K. H. Erlwanger. "Neonatally administered zingerone protects against high-fructose diet-induced dyslipidaemia and visceral obesity in male Sprague-Dawley rats". 10th Cross-Faculty Postgraduate Symposium of the University of the Witwatersrand, Johannesburg, South Africa (3rd - 4th September 2019). The poster was awarded the First Prize Winner (First Round), and the Third Prize Winner (Second Round).
2. **N. Muhammad,** B. W. Lembede and K. H. Erlwanger. "Neonatally administered zingerone prevents high dietary fructose-induced hepatic steatosis in Sprague Dawley rats". Faculty of Health Sciences Biennial Research Day, University of the Witwatersrand, Johannesburg, South Africa (15th October 2020).

PUBLICATIONS ARISING FROM THIS RESEARCH PROJECT

Published manuscripts

1. **Muhammad N**, Lembede B.W, and Erlwanger K.H. (2020). Neonatal zingerone protects against the development of high-fructose diet-induced metabolic syndrome in adult Sprague-Dawley rats. *Journal of Developmental Origins of Health and Disease*, pp. 1-9. doi: 10.1017/S2040174420000525

2. **Muhammad N**, Lembede B.W, and Erlwanger K.H. (2021). Zingerone administered neonatally prevents the subsequent development of high dietary fructose-induced fatty liver in Sprague Dawley rats. *Journal of Medicinal Food*, pp 1-9. doi: 10.1089/jmf.2020.0189

Manuscripts currently under review

1. **N. Muhammad,** B.W. Lembede and K.H. Erlwanger. (2021). Neonatal administration of zingerone prevents the subsequent development of high dietary fructose-induced early features of nephropathy in rats. Submitted to the Journal of *Animal Physiology and Animal Nutrition* (Manuscript ID: JAPAN-Apr--21-285).

ABSTRACT

Management of metabolic disorders places a heavy burden on healthcare systems globally. Dietary manipulations during developmentally plastic periods including the early postnatal phase can result in long-term beneficial or adverse health outcomes. Consumption of high-fructose diets early in life increases the risk of developing metabolic syndrome and associated cardiovascular and renal complications. Zingerone, a phytochemical mainly isolated from ginger (*Zingiber officinale)*, has been demonstrated to attenuate metabolic derangements in adult rats. The potential preventive effects of zingerone administered orally to neonatal male and female rats against the long-term development of high-fructose diet-induced metabolic derangements were investigated.

Four-day old male and female Sprague Dawley rat pups (n = 79) were randomly grouped and gavaged with: 10 ml/kg body weight of distilled water (W), 10 ml/kg body weight 20% fructose solution (FS), 10 ml/kg body weight fructose solution + 40 mg/kg body weight of zingerone in distilled water (ZF), or 40 mg/kg body weight of zingerone in distilled water (ZW) pre-weaning. After weaning, W and ZW continued on unlimited tap water while FS and ZF continued on unlimited fructose solution for 10 weeks. Commercial rat feed was provided *ad libitum*. Food and fluid intake was evaluated. Blood samples were collected for metabolic assays and assessment of general health markers. Growth performance, adiposity, hepatic lipid accumulation, renal function pathology and gastro-intestinal tract (GIT) organs' morphometry were assessed. Liver and kidney tissues were collected for histological evaluation.

Food intake was decreased; overall caloric intake was increased due to fructose feeding in both sexes ($P < 0.05$; ANOVA). When compared with the negative controls, the high-fructose diet significantly raised the terminal body masses [Females ($P < 0.0001$; ANOVA)], body mass index (BMI) [Females ($P = 0.0036$; ANOVA)], concentrations of triglycerides, total cholesterol, low density lipoprotein cholesterol, triglycerides to high density lipoprotein cholesterol ratio, visceral fat mass relative to body weight [Both sexes ($P < 0.05$; ANOVA)] and empty carcass mass [Females ($P = 0.0025$; ANOVA)]. Neonatally administered zingerone prevented ($P < 0.05$; ANOVA) the fructose-induced increase in body mass and empty carcass mass (Females), and hypercholesterolemia (Both sexes). Lee index and glycaemic parameters were not affected by the interventions in both sexes ($P > 0.05$; ANOVA).

Rats on the high-fructose diet compared to the negative controls had significantly increased hepatic lipid content [(%), $P = 0.0002$ (Males), $P < 0.0001$ (Females); ANOVA] and hepatic

steatosis score [(%), $P = 0.0018$ (Males), $P < 0.0022$ (Females); Kruskal-Wallis ANOVA]. Zingerone administered neonatally prevented ($P < 0.05$; Kruskal-Wallis ANOVA) the fructose-induced increase in hepatic steatosis in both sexes. The plasma levels of uric acid, markers of liver function, lipid peroxidation and inflammation were not different ($P > 0.05$; ANOVA) across the different treatment groups in both sexes.

The group administered fructose only had significantly [$P = 0.0054$ (Males), $P = 0.0002$ (Females); ANOVA] increased levels of kidney injury molecule 1 (KIM-1), and decreased urinary space area [$P = 0.0001$ (Males), $P = 0.0016$ (Females); ANOVA] compared to the controls. Neonatally administered zingerone prevented the fructose-induced increase in the levels of KIM-1 [$P = 0.9262$ (Males), $P = 0.6667$ (Females); ANOVA], and fructose-induced reduction in the urinary space area [$P = 0.1505$ (Males), $P = 0.8265$ (Females); ANOVA] when the combined fructose and zingerone administered group was compared with the negative controls.

Sex related differences were observed in food, fluid and caloric intake, terminal mass, BMI, cholesterol subtypes, visceral fat percentage, GIT visceral organs and long bones' morphometry and empty carcass mass ($P < 0.05$; ANOVA).

In rats, zingerone can be used strategically in the neonatal phase for prophylactic management of long-term high-fructose diet-induced metabolic syndrome, non-alcoholic fatty liver disease and nephropathy. Future studies in human clinical trials should be undertaken to explore the applicability of these findings to reduce the burden of metabolic disease on healthcare systems.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisors**, Professor Kennedy Honey Erlwanger and Dr Busisani Wiseman Lembede** for their kind and professional supervision.

My gratitude also goes to **the staff of the Wits Research Animal facility (previously known as the Central Animal Service)**, Faculty of Health Sciences, University of the Witwatersrand, for their assistance with the care and welfare of the animals.

Special thanks to **Dr Trevor Nyakudya** for his technical support and assistance during termination, and **Miss Amelia Rammekwa** for her technical assistance with the analysis of samples on the VetLab chemistry analyser.

I appreciate the technical assistance of **Miss Monica Gomes** in performing metabolic assays and histology. **Miss Hasiena Ali** (School of Anatomical Sciences) is also acknowledged for her technical assistance with the histology images.

My postgraduate colleagues cannot be forgotten for their assistance with terminal procedures: **Miss Thobekile Precious Dladla, Mr Bayanda Mdoda, Mr Austin Azubike Ajah and Miss Nonosi Kennedi.**

The support, patient, inspiration and encouragement of **my wife**, and the interruptions from my **four-month-old daughter**, are thankfully acknowledged.

I highly appreciate the financial support received from various bodies for this research. These bodies included the **Medical Faculty Research Endowment Fund, Faculty of Health Sciences Research Committee and School of Physiology of the University of Witwatersrand (Grant Number: 001 254 8521101 5121105 000000 0000000000 5254), and National Research Foundation (NRF) Thuthuka Fund (Grant Number: TTK170415227205).** The **Federal University Birnin Kebbi (Nigeria) and Tertiary Education Trust Fund of Nigeria** are also acknowledged for supporting the PhD candidate.

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LIST OF ABBREVIATIONS

AACE: American Association of Clinical Endocrinologists

ACC-1: Acetyl Coenzyme A carboxylase 1

Acetyl-CoA: Acetyl coenzyme A

ADPT: Adaptation

AESC: Animal Ethics Screening Committee

AHA: American Heart Association

ALT: Alanine aminotransferase

AMP: Adenosine monophosphate

ANOVA: Analysis of variance

AOAC: Association of Official Agricultural Chemists

AREC: Animal Research Ethics Committee

ARRIVE: Animal Research: Reporting of *In Vivo* Experiments

ATP: Adenosine triphosphate

ATP-III: Adult Treatment Panel Three

BMI: Body mass index

bwt: Body weight

C: Negative control group (in chapter 4)

CA: Corpuscular area

cAMP: Cyclic adenosine monophosphate

CAS: Central Animal Service

ChREBP: Carbohydrate responsive element binding protein

CI: Confidence interval

Con: Negative control group (in chapter 5)

d: Difference between mean values

DNA: Deoxyribonucleic acid

DNL: *de novo* lipogenesis

DOHaD: Developmental Origins of Health and Disease

EGIR: European Group for the study of Insulin Resistance

ELISA: Enzyme-Linked Immuno-Sorbent Assay

F: Females

FBG: Fasting blood glucose

Fig: Figure

Fr: The rat group that drank only 20% fructose solution (in chapter 4)

Fru: The rat group that drank only 20% fructose solution (in chapter 5)

FS: The rat group that drank only 20% fructose solution (in chapters 3 and 6)

G.D: Glomerular density

GFR: Glomerular filtration rate

GIT: Gastrointestinal tract

GLUT: Glucose transporter

GTA: Glomerular tuft area

H_0: Null hypothesis

H_1: Alternative hypothesis

H & E: Haematoxylin and eosin

Hb: Haemoglobin

HDL: High-density lipoprotein

HDL-c: High-density lipoprotein cholesterol

HFCS: High fructose corn syrup

HIV: Human immunodeficiency virus

HOMA-IR: Homeostatic model of insulin resistance

IAS: International Atherosclerosis Society

IASO: International Association for the Study of Obesity

IDF: International Diabetes Federation

IL-6: Interleukin 6

KIM-1: Kidney injury molecule 1

LD: Lethal dose

LDL: Low-density lipoprotein

LDL-c: Low-density lipoprotein cholesterol

M: Males

MAFLD: Metabolic dysfunction-associated fatty liver disease

MCHC: Mean corpuscular haemoglobin concentration

MDA: Malondialdehyde

mRNA: Messenger ribonucleic acid

MT: Masson's trichrome

n: Number

NACEP: National Cholesterol Education Program

NAFLD: Non-alcoholic fatty liver disease

NAFPD: Non-alcoholic fatty pancreas disease

NASH: Non-alcoholic steatohepatitis

NEFA: Non-esterified fatty acids

NHLBI: National Heart, Lung and Blood Institute

OGTT: Oral glucose tolerance test

***P*:** Predictive value

PCV: Packed cell volume

PND: Postnatal day

PPAR: Peroxisome proliferator-activated receptor

PSSA: Physiology Society of Southern Africa

R.D: Relative density

RNA: Ribonucleic acid

ROS: Reactive oxygen species

SANS: South African National Standard

SD: Standard deviation

SRC: Standard rat chow

SREBP-1: Sterol regulatory element-binding protein 1

STZ: Streptozotocin

TAG: Triacylglycerol

TBARS: Thiobarbituric acid reactive substances

TC: Total cholesterol

TGs: Triglycerides

TNF: Tumour necrosis factor

USA: Urinary space area

VLDL: Very low-density lipoprotein

VLDL-c: Very low-density lipoprotein cholesterol

W: Negative control group (in chapters 3 and 6)

WC: Waist circumference

WHF: World Heart Federation

WHO: World Health Organisation

Z: The group that was administered only zingerone (in chapter 4)

ZF: The group administered/drank combined fructose and zingerone (in chapters 3 and 6)

ZFr: The group administered/drank combined fructose and zingerone (in chapter 4)

ZFru: The group administered/drank combined fructose and zingerone (in chapter 5)

Zgr: The group that was administered only zingerone (in chapter 5)

ZW: The group that was administered only zingerone (in chapters 3 and 6)

AUTHOR CONTRIBUTIONS

The study concept and design were effected after having discussions with my primary and co-supervisors, Professor Kennedy H. Erlwanger and Dr Busisani W. Lembede respectively. With the help of my supervisors, I conducted the experimental procedures, collected, analysed and interpreted the data, and wrote the first draft of the manuscripts and thesis. My supervisors helped with the planning and implementation of the study, collection and interpretation of the data, and editing of the thesis and constituent manuscripts.

As supervisors of the candidate, we hereby confirm that Nasiru Muhammad has described the roles played by his co-authors precisely and that he functioned as the principal investigator of the study.

Prof. Kennedy H. Erlwanger……  .....Date……02/06/2021.....

Dr. Busisani W. Lembede…….  .....Date……02/06/2021……

CHAPTER ONE - Introduction and justification

1.1 Thesis structure

This thesis is structurally presented in a divided block format, and consists of eight chapters as well as a section on appendices. At the end of each chapter, a list of all the references used in that chapter is presented.

Chapter 1 provides the structure of the thesis, and an overview of the research problem, which fundamentally encompasses early life environmental and nutritional influences on the long-term development of metabolic disorders, dietary fructose-induced metabolic disorders, the global burden associated with the management of metabolic disorders and the need for alternative management strategies using medicinal plants and phytochemicals derived from them. The chapter also provides the justification, main aim, objectives and hypotheses of the study.

A critical review of the literature relevant to this study has been provided in Chapter 2. This includes definitions, global prevalence, pathophysiology and risk factors associated with the development of metabolic syndrome and its complications, not only in adults but also in the paediatric age group. The chapter also describes the role of fructose in the pathogenesis of metabolic disorders, the available therapeutic options and their drawbacks, and the need for alternative, safe and readily available management options for fructose-induced metabolic dysfunction using plant phytochemicals. Furthermore, this chapter also discusses the importance of zingerone, a phytochemical from the plant ginger, which has a variety of biological and pharmacological actions.

The role of neonatal programming in the pathogenesis of long-term metabolic dysfunction is also introduced in this chapter, with special emphasis on the early postnatal period of development as a potential target for prophylactic and therapeutic approaches in the management of fructose-induced metabolic dysfunction with a specific focus on non-alcoholic fatty liver disease, general metabolism and nephropathy

The findings of the current study are presented in Chapters 3, 4, 5 and 6, and each of these chapters is presented in a format that consists of the following main sections: introduction, materials and methods, results, discussion and conclusion. Chapters 3 and 4 are published manuscripts, whilst chapter 5 is a submitted manuscript. However, Chapter 6 is not a published/submitted manuscript.

In Chapter 3, we investigated the effects of neonatally administered zingerone on the development of high-fructose diet-induced metabolic syndrome in adult male and female Sprague Dawley rats. In this chapter, the features of general metabolism including body mass gain, visceral adiposity and clinical metabolic parameters in circulation; in addition to food, fluid and calorie intake were explored. The findings from this chapter were published in the peer-reviewed ISI-accredited journal: *Journal of Developmental Origins of Health and Disease*.

Chapter 4 focuses on the development of non-alcoholic fatty liver disease (NAFLD) secondary to excess fructose consumption by male and female Sprague Dawley rats, and the potential long-term protective effects of neonatally administered zingerone on the NAFLD. These included the determination of hepato-somatic index, hepatic lipid accumulation, histomorphometry of the liver, key liver enzyme, uric acid and markers of oxidation and inflammation. The findings from this chapter were published in the internationally accredited and peer-reviewed *Journal of Medicinal Food.*

The potential long-term protective effect of neonatal oral administration of zingerone against the subsequent development of nephropathy in fructose-fed male and female Sprague Dawley rats was investigated and presented in Chapter 5. These included determination of kidney mass, kidney injury molecule 1, kidney tissue antioxidant and histomorphometric evaluation of the kidneys. The findings from this chapter were submitted to the internationally accredited and peer-reviewed Journal of *Animal Physiology and Animal Nutrition* , and are currently under review.

Chapter 6 is in essence a supplementary chapter in which pertinent data collected from the study but not included in the preceding chapters is contained. Thus, the effect of neonatal administration of zingerone on further measures of general health of the fructose-fed male and female Sprague Dawley rats is presented. The chapter reports on the obesity and red blood cell indices, gastrointestinal tract (GIT) visceral organ morphometry, masses of heart and pancreas, long bones' morphometry and empty carcass mass.

In Chapter 7, an overall discussion of the thesis based on chapters 3, 4, 5 and 6 is presented, in which all the findings of the individual chapters are articulated and contextualised.

Chapter 8 recapitulates, in summary, the major findings and then identifies limitations of the study and proffers recommendations for future studies. The key conclusions are also drawn. The Appendices section consists of supplementary Figures and Tables from Chapter 3, a copy of the institutional documents pertinent to ethics for the study, namely the ethical clearance certificate and ethical modification and extension forms. The plagiarism declaration as well as further details on methodology [Soxhlet procedure, Enzyme-Linked Immunosorbent Assay (ELISA) and IDEXX procedures] are included. The printed versions of the published manuscripts and the signed copyright agreement forms from the respective journals are also included.

1.2 Background and study justification

The early postnatal period is a developmentally plastic stage of life in which nutritional influences can result in genetic/epigenetic changes that render the growing neonate susceptible or resistant to diseases [1]. Nutrition during the early developmental stages can lead to metabolic dysfunction immediately or later in life, a concept described as "metabolic programming" by multiple hypotheses [2,3]. Single nutritional hit during the early developmental stages leads to metabolic dysfunction immediately or later in life (single hit hypothesis) [2]. Following a perinatal nutritional hit (first single hit), the risk of developing metabolic derangements is increased after a second nutritional hit is introduced later in life (double hit hypothesis) [3]. In addition, predisposition to metabolic programming involves several other factors (hits) that act together in genetically predisposed individuals (multiple hit hypothesis) [4].

Excessive intake of high fructose diets during the perinatal period may serve as first hit that may lead to subsequent multiple hits thereby triggering the development of metabolic diseases including obesity, non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome later in life [4]. Metabolic syndrome is a concomitant presentation of several metabolic disorders including central obesity, dyslipidaemia, hyperglycaemia and NAFLD [5], and can be complicated by cardiovascular [6], nephrological [7] and haematological [8] involvement. The global prevalence of metabolic disorders is estimated at 20-25% in the adult population [9]; with females accounting for 52% of the prevalence [10]. Certain genes, receptors and metabolic hormones are involved in fructose metabolism, and these may be affected by dietary changes in the early postnatal period leading to adverse metabolic programming impacting health later in life [1].

The long-term development of metabolic disorders due to nutritional influences in the early developmental period of plasticity has been attributed to immaturity of the physiological systems of neonates [11,12]. For example, the differentiating satiety centre in the brain that controls appetite and nutrition, is partly influenced by intrinsic and extrinsic factors as it is not fully matured during the early postnatal period [13]. As such, consumption of dietary food substances (including fructose) during the early postnatal period can programme for precocious maturation of organs and alteration of physiological processes [14]. This increases the global prevalence of obesity and metabolic syndrome [15], not only in adults but in growing children, thereby increasing the incidence of obesity, type 2 diabetes and cardiovascular complications in adult life [16].

Babies may consume excess fructose via infant feeding formulas during the neonatal period due to insufficient breast milk from the mother, or when the mother chooses not to breastfeed, or in disease conditions of mothers that require limited breastfeeding like Human Immunodeficiency Virus (HIV) infection [17,18]. These infant formulas may contain a large amount of fructose, which can cause long-term development of metabolic disorders [19]. Thus, there is a need to investigate the long-term metabolic health consequences of early life fructose consumption that mimic the excess fructose consumption by babies.

The management of metabolic disorders has become a global challenge due to the greater global economic burden of managing metabolic syndrome [6]. The high cost of using synthetic conventional therapy makes the drugs inaccessible to the majority of the populace [20]; the associated drug adverse side effects and drug reactions secondary to prolonged and combination therapy have also made the conventional therapy uncomfortable [21]. This makes the majority of the global communities to prefer plant-derived medications and phytochemicals for their healthcare problems [21]. The World Health Organisation (WHO) has encouraged the exploration of natural plants for medicinal purposes [22]. As such, an interest in the strategic use of medicinal plants and naturally-derived phytochemicals with a broad spectrum of biological action is growing [21]. Previous studies have demonstrated beneficial health outcomes with the use of phytochemicals in the management of metabolic disorders. These phytochemicals include bergenin [23], S-allyl cysteine [24], malonyl ginsenosides [25] and oleanolic acid [26]. Therefore, to expand on this knowledge, the current study strategically selected zingerone as a candidate phytochemical. Zingerone is a semi water-soluble alkaloid found in the plant ginger (*Zingiber*

officinale) [27]. *In vitro* and *in vivo* studies have reported beneficial metabolic health outcomes associated with zingerone in adult rat models [28–30]. The mechanisms by which zingerone exerts health beneficial effects include preservation of pancreatic β-cells and upregulation of lipolysis in adipose tissues [29].

Oral administration of dietary food substances and phytochemicals during the neonatal period affect the digestive, absorptive or regulatory capacity of the GIT [31]. For example, consumption of excess fructose diets during the neonatal period has been shown to alter the expression of glucose transporter 5 (GLUT-5) in the GIT leading to adverse metabolic programming [32,33]. Phytochemicals that are orally ingested during the neonatal period can alter the development of the gut and its accessory organs, thereby affecting the GIT morphometry and function, which in turn affects the growth of the growing offspring [14]. In addition, dietary interventions may adversely affect other vital organs including the heart, kidney [15] and reproductive organs [34]. Thus, there is a need to evaluate the impact of dietary interventions on general health. The majority of the studies that explored the metabolic effects of fructose or zingerone have been performed in adult male rat models after the weaning period [27,35–39]. There is a paucity of information from animal studies involving fructose or zingerone administration during the neonatal period using mixed gender. Thus, it is pertinent to investigate the long-term effects of neonatally administered zingerone and fructose in both male and female rat models.

In summary, the global increasing prevalence of fructose-induced metabolic disorders has a chronic debilitating impact on the metabolic health of children and adolescents [40]. Conventional therapy is not readily available to most global communities [20]. The impact of dietary interventions on metabolic and general health is being recognised [41–43]. Sex and gender variations have also been reported regarding the developmental programming of metabolic disorders [12]. Therefore, this study investigated the long-term preventive effects of neonatally administered zingerone on fructose-induced metabolic dysfunction in growing male and female Sprague Dawley rats.

1.3 Aims and objectives

The main aim of the study was to determine the potential of orally administered zingerone in neonates to protect against the development of metabolic derangements, in adolescence, induced by high-fructose diet.

The specific objectives of the study were to determine whether zingerone could confer long-term protection against the adverse outcomes of health and metabolism of high-fructose diet in male and female Sprague Dawley rats by specifically determining zingerone's effects on four focus areas:

i. Growth performance

By determining/measuring:

a) fluid and feed intake
b) whole body and empty carcass masses, body length, body mass index (BMI), obesity index and linear growth

ii. General metabolism and general health

By determining/measuring:

a) fasted blood glucose, triglycerides (TGs) and cholesterol [total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c) concentrations, and uric acid
b) erythrocyte packed cell volume (PCV), haemoglobin (Hb) concentration and mean corpuscular haemoglobin concentration (MCHC)
c) selected hormones that regulate metabolism (insulin and adiponectin) and whole-body insulin sensitivity by computing the homoeostasis model assessment of insulin resistance (HOMA-IR index)
d) antioxidant status [thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA)]
e) inflammatory mediators [interleukin six (IL-6) and tumour necrosis factor-alpha (TNF-α)
f) morphometry of visceral organs (stomach, small intestines, large intestines and caecum)
g) masses of heart, pancreas and testes (males)
h) measures of obesity [visceral fat and epididymal fat pad (males) masses]

iii. Non-alcoholic fatty liver disease

By measuring/evaluating:

a) surrogate markers of liver function (alanine aminotransferase)
b) liver mass
c) liver lipid content

d) liver histology

iv. *Nephropathy*

By measuring/evaluating:

a) kidney mass
b) kidney histomorphometry
c) surrogate markers of kidney function [kidney injury molecule one (KIM-1)]

1.4 Hypotheses

H_1: Oral administration of zingerone to neonatal male and female Sprague Dawley rats confers protection in adolescence against the development of metabolic derangements induced by long-term consumption of a fructose-rich diet.

H_0: Oral administration of zingerone to neonatal male and female Sprague Dawley rats does not confer protection in adolescence against the development of metabolic derangements induced by long-term consumption of a fructose-rich diet.

The following chapter provides a detailed critical review of the literature relevant to the current study. The chapter covers the concepts of neonatal metabolic programming and metabolic syndrome, and their long-term effects in adolescent life. It also describes the beneficial effects of zingerone on metabolic and general health.

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CHAPTER TWO - Literature review

2.1 Metabolic syndrome

2.1.1 Introduction

Metabolic disorders are derangements or diseases resulting from abnormal metabolic processes [1]. They include central obesity, dyslipidaemia, insulin resistance, hyperglycaemia, hypertension and non-alcoholic fatty liver disease (NAFLD); and these derangements constitute a clinical term known as metabolic syndrome, which may be complicated by cardiovascular diseases and type 2 diabetes [2,3]. The prevalence of metabolic syndrome grows continuously, and it is estimated that more than one billion people of the world population are affected by the syndrome [4].

2.1.2 Prevalence of metabolic syndrome

The global prevalence of metabolic syndrome in the adult population is approximately 20-25% [5]; in which the prevalence is 51.7-53% in females [4]. This prevalence varies according to race/ethnicity [5] and region of the world. For instance, in the United States, the prevalence of metabolic syndrome is 33.4%, whilst 14.4% in China [5]. Additionally, in the United States alone, the prevalence is lowest in African-Americans but highest in Hispanics [5]. In 2017, it was reported that the prevalence of metabolic syndrome in South Africa was 21.8%; with males having a higher prevalence of 24.8% than females with 15.6% [6].

The increasing prevalence of metabolic syndrome has a deleterious impact on the global health economic burden [2,7]. The total global cost of managing metabolic syndrome and its complications and potential decrease in economic activity has been estimated to be in trillions of dollars [4]. Metabolic syndrome is defined in different ways using various criteria [8].

2.1.3 Definitions of metabolic syndrome

Attempts to define metabolic syndrome began long ago as a concept instead of diagnosis in 1923, when a Swedish physician named Kylin, reported that an association exists between elevated blood pressure, elevated blood glucose levels and elevated levels of uric acid [9]. This observation was followed by that of Vague in 1947, who demonstrated that visceral adiposity was invariably associated with the development of metabolic derangements seen in cardiovascular diseases and type 2 diabetes [10]. In 1965, Avogaro and Crepaldi presented an abstract at the annual meeting of the European Association for the Study of Diabetes, where they described it (metabolic syndrome) as a syndrome that comprised of obesity, hyperglycaemia and hypertension [11]. Significant development was made by Reaven in 1988 when he introduced the

idea of insulin resistance and called it “Syndrome X“ that was associated with diabetic and cardiovascular risk factors [12]. However, at that time Reaven did not include obesity in his definition of metabolic syndrome. In 1989, the syndrome was named “The Deadly Quartet” by Kaplan for recognizing a combination of obesity, glucose intolerance, hypertriglyceridaemia, and hypertension [13]; and this was later renamed in 1992 as “The Insulin Resistance Syndrome” by Haffner et al. [14] because they believed that insulin resistance is the basic underlying pathology in the development of metabolic syndrome.

Based on the above developments, various international organisations have developed different diagnostic criteria for metabolic syndrome in subsequent years. These organisations include the World Health Organization (WHO) in 1998 [15], the European Group for the study of Insulin Resistance (EGIR) in 1999 [16], the National Cholesterol Education Program-Third Adult Treatment Panel (NCEP:ATP III) in 2001 [17] and American Association of Clinical Endocrinologists (AACE) in 2003 [18]. However, none of these organisations completely considered all the major factors that affect the prevalence of metabolic syndrome including age, sex, race and ethnicity [19]. This has made the diagnosis of metabolic syndrome clinically and epidemiologically difficult across different populations globally [19]. Additionally, the previous definitions could not easily identify those people at risk of developing cardiovascular diseases and type 2 diabetes [19].

As a result, the International Diabetes Federation (IDF) collaborated with the representatives who came from different parts of the world, including previous organisations that had developed earlier definitions [20]. These representatives came up with a new definition of metabolic syndrome that harmonised the previous criteria together, which can be applied to different populations of the world. The new definition gives an easy way of clinically identifying people at risk of developing type 2 diabetes and cardiovascular diseases, in addition to enhancing epidemiological research into the topic of metabolic syndrome [20–22]. Table 2.1 shows the new IDF criteria for the diagnosis of metabolic syndrome in adults.

Table 2.1: The new IDF criteria for the diagnosis of metabolic syndrome in adults

Criteria	**Reference values**
Central obesity* or BMI > 30 kg/m^2	WC ≥ 90-94 cm (♂) WC ≥ 80 cm (♀)
Dyslipidaemia	Triglycerides ≥ 1.7 mmol/L (or specific treatment for the abnormality)
	HDL-c < 1.03 mmol/L (♂) HDL-c < 1.29 mmol/L (♀) (or specific treatment for the abnormality)
Hypertension	Systolic blood pressure ≥ 130 mm Hg or Diastolic blood pressure ≥ 85 mm Hg (or treatment of previously diagnosed hypertension)
Fasting glucose	≥ 5.6 mmol/L (or previous diagnosis of type 2 diabetes)

* *= major criteria (with ethnicity-specific values) that must be present, plus any other two or more criteria; IDF = international diabetes federation; WC = waist circumference; BMI = body mass index; HDL-c = high-density lipoprotein cholesterol; ♂ = males; ♀ = females. Table modified from Alberti et al.* [20].

In 2009, some organisations came up with a modified IDF definition of metabolic syndrome after reaching a consensus that, central obesity alone should not be considered as the major component, rather all the five components identified in the IDF definition should equally be recognised as major components, in addition to elevated levels of low-density lipoprotein-

cholesterol (LDL-c). These organisations include the American Heart Association (AHA), the National Heart, Lung, and Blood Institute (NHLBI), the World Heart Federation (WHF), the International Atherosclerosis Society (IAS), the International Association for the Study of Obesity (IASO) and the IDF [21–23]. Representatives from these organisations unanimously agreed that the presence of any three out of the above six components is diagnostic of metabolic syndrome [22].

2.1.4 Components of metabolic syndrome

Central obesity, dyslipidaemia, insulin resistance and hyperglycaemia are amongst the major components of metabolic syndrome [2]. Globally, more than two billion of the adult population are overweight, in which more than 650 million are obese, with males and females accounting for 11% and 15% respectively [24]. Obesity, a resultant of dyslipidaemia, is a clinical condition characterized by an excess amount of fat tissue manifesting with an abnormal increase in body mass index (BMI) [25] and or a Lee index [26]. As the global prevalence of obesity gradually increases, likewise the growing burdens of its associated metabolic derangements including insulin resistance and hyperglycaemia [24]. Abnormal increase in circulating free fatty acids, LDL-c and triglycerides, with an associated decrease in HDL-c, can induce insulin resistance thereby preventing glucose transport and uptake in the peripheral tissues leading to overt hyperglycaemia [27]. Additionally, obesity is invariably associated with NAFLD [3].

In addition to the aforementioned components of metabolic syndrome, it has recently been shown that NAFLD is also a component of metabolic syndrome due to its association with obesity, dyslipidaemia, insulin resistance, hyperglycaemia or any metabolic derangements; and that is why NAFLD is now called metabolic dysfunction-associated fatty liver disease (MAFLD) [28]. The inclusion of NAFLD into the components of metabolic syndrome was proposed by a panel of international experts from different countries [28]. Obesity is the driving force for the development of metabolic derangements [24].

The prevalence of NAFLD is higher in males compared to females and it increases with age [29], and is estimated at 25% globally [30]. NAFLD occurs when hepatocytes are infiltrated with lipid droplets without significant alcohol consumption [31]. It can present as simple steatosis and then progresses to non-alcoholic steatohepatitis (NASH) [31]. NASH is characterized by inflammation and progressive fibrosis [32].

The aforementioned components of metabolic syndrome are attributed to various causes and risk factors, which are discussed briefly in the following sections.

2.1.5 Aetiology, risk factors and pathogenesis of metabolic syndrome

The exact cause of metabolic syndrome is not well understood, however, several risk factors and mechanisms have been implicated, and these include adipose tissue dysfunction, insulin resistance, chronic inflammation, oxidative stress, gut microbiota, genetics/epigenetics [2], poor dietary habits and a decrease in physical activity [33]. Central obesity is believed to be the initiator of many different abnormal metabolic pathways that lead to the development of full-blown metabolic syndrome [34].

2.1.5.1 Adipose tissue dysfunction

Obesity, defined as excess fatty tissue mass, is a key driving factor in the pathogenesis of insulin resistance, hyperglycaemia and metabolic syndrome [35,36]. The increase in fat mass could be due to adipocyte hypertrophy and hyperplasia secondary to overfeeding with high-calorie diets [36]. The accumulated fat can change its biological characteristics and adopt lipolysis that leads to the release of fatty acids, increased LDL-c and decreased HDL-c levels, features that are invariably associated with metabolic syndrome [2]. Central (visceral) obesity is more indicative of metabolic syndrome [20,37], because rates of lipolysis and susceptibility to apoptosis is high in visceral adipocytes [36].

Obesity is also characterized by recruitment and infiltration of inflammatory and immune cells into adipose tissue resulting in increased secretion of pro-inflammatory cytokines [such as interleukin six (IL-6) and tumour necrosis factor-alpha (TNF-α)], and immune cells (primarily macrophages) [35]. These cells are all typically involved in the pathogenesis of insulin resistance, and eventually, type 2 diabetes [35].

Adiponectin is an anti-inflammatory cytokine secreted by adipose tissue, and the circulating levels of adiponectin are inversely related to the concentrations of fasting plasma glucose and insulin, and visceral fat accumulation [21]. Adiponectin increases insulin sensitivity and enhances energy metabolism and fatty acid oxidation by activating peroxisome proliferator-activated receptor alpha (PPARα) [38,39]. Thus, low adiponectin concentrations that are associated with metabolic syndrome translate to decreased insulin sensitivity (insulin resistance), elevated fasting

blood glucose (leading to type 2 diabetes), and increased tissue triglycerides which can lead to the pathogenesis of NAFLD [40].

Additionally, the pro-inflammatory cytokines can affect the heart and its vasculature thereby playing a possible role in adiposity-related inflammation and atherosclerosis, and eventual cardiovascular dysfunction [36]. Furthermore, obesity is invariably associated with increased oxidative stress [37].

2.1.5.2 Oxidative stress

Oxidative stress results from an imbalance between the production and degradation of reactive oxygen species (ROS), which are produced in the mitochondria from the oxygen utilised for oxidative phosphorylation through the electron transport chain [41]. In normal situations, the generated ROS can be balanced out by the individual's innate antioxidants' systems, however, excess high-calorie diet intake can increase the mitochondrial metabolic load leading to excessive electron transport chain activity that can form excessive ROS, which contributes to mitochondrial damage, affecting normal cellular and metabolic processes [2]. ROS serves as the trigger as well as an outcome of obesity. Abnormal generation of ROS can induce adipogenesis and therefore contributes to the development of obesity and metabolic syndrome [42]. Conversely, obesity can cause systemic oxidative stress through endoplasmic reticulum stress in adipocytes, excessive ROS production (after high-calorie diet) and suppression of anti-oxidative system [21]. These processes contribute to the development of metabolic disorders including diabetes and cardiovascular diseases [2].

Despite adiponectin being an antioxidant cytokine that acts as an anti-atherogenic and vasodilator [21], the expression of this cytokine (adiponectin) is downregulated in metabolic syndrome. This leads to the generation of ROS, thereby activating an oxidative cascade that leads to programmed cell death, cellular destruction, breached endothelial cell integrity and eventual atherosclerosis and cardiovascular dysfunctions [21].

Additionally, the elevated levels of ROS can cause insulin resistance via induction of beta-cell dysfunction [2].

2.1.5.3 Insulin resistance

Insulin is a hormone secreted by pancreatic beta cells, which mainly regulates glucose metabolism [43]. It is released in response to an increase in blood glucose levels after feeding [2]. Insulin stimulates the uptake of blood glucose into peripheral tissues (mainly via GLUT 4

transporter) for glycolysis, or storage as glycogen in the hepatocytes, myocytes, and/or adipocytes for future requirements [21]. This process suppresses hepatic gluconeogenesis and glycogenolysis [43]. The aim of these physiological processes is to maintain normal blood glucose level [2].

In an insulin-resistant situation, initial insulin secretion is lost in response to a glucose load, resulting in postprandial hyperglycaemia; and subsequent exaggerated insulin response causes chronic hyperinsulinaemia [20]. Since insulin-responsive tissues cannot respond to insulin efficiently, insulin-mediated glucose uptake, glycolysis, and glycogen synthesis are all impaired [43]. Thus, insulin resistance worsens gradually and pancreatic beta cells become stressed, fatigued and apoptotic, until they completely lose their insulin-secreting function leading to diabetes mellitus and metabolic syndrome [2].

Insulin also stimulates fatty acid synthetic pathways via decreasing the activity of cyclic adenosine monophosphate (cAMP) [44]. Accumulation of lipids when energy intake outweighs storage capacity may also contribute to increased delivery of free fatty acid to the tissues [45]. Circulating free fatty acids are derived mainly from adipose tissue triglyceride stores through the action of cAMP during lipolysis [46]. Within the hepatocytes, free fatty acids enhance the increased synthesis of triglycerides and triglyceride-rich very-low-density lipoproteins, which are atherogenic [21]. When the effects of insulin are decreased during insulin resistance states, there will be an increase in the rate of lipolysis, leading to increased free fatty acid production [43]. This will further inhibit the lipogenic properties of insulin, leading to further lipolysis, which contributes to atherogenic dyslipidaemia, hypertension and metabolic syndrome [21].

2.1.5.4 Chronic inflammation

Adipose tissue is both an endocrine and paracrine organ that is biologically active [21]. In situations of excess high-calorie diets in which excess fat is accumulated, adipocytes undergo hypertrophy and hyperplasia thereby increasing blood requirement of the cells, with subsequent generation of a hypoxic state [21]. The hypoxia sends signals that attract monocytes in circulation and recruit them to local adipose tissue, where they (monocytes) differentiate to macrophages [47]. This leads to cell necrosis, infiltration of macrophages and adipocytokines production including IL-6 and TNF-α [2,21].

These cytokines (IL-6 and TNF-α) generate local adipose inflammatory responses which contribute to local insulin resistance, with subsequent generation of systemic inflammation and systemic insulin resistance leading to the development of metabolic syndrome [2]. IL-6 is a pro-inflammatory cytokine that plays a key role in the pathogenesis of insulin resistance and type 2 diabetes [21]. Increased levels of IL-6 have been associated with obesity, atherosclerosis, hypertension and cardiovascular dysfunctions [2]. TNF-α is also a pro-inflammatory cytokine that acts as a paracrine mediator to several cardiovascular disorders, including atherosclerosis, hypertension and heart failure [21].

2.1.5.5 Genetics and epigenetics

It has been reported that genetic factors interact with the environment and contribute to the development of metabolic syndrome [2]. Whilst some genes are known to be associated with obesity and metabolic syndrome, epigenetics plays a key role in promoting the development of metabolic syndrome than genetic predisposition [48]. It has been shown that postnatal dietary manipulations are strongly correlated with the development of metabolic syndrome in adulthood [49]. The mechanism implicated for this phenomenon is either hypo- or hypermethylation of deoxyribonucleic acid (DNA) [50].

Metabolism of carbohydrates (fructose inclusive) depends on the expression of genes and receptors which include sterol regulatory element-binding protein 1 (SREBP-1), acetyl-CoA carboxylase 1 (ACC-1), carnitine palmitoyltransferase 1 (CPT1), TNF-α and PPARα [50]. The expression of these genes is affected by DNA methylation, the pattern of which may be affected by changes in diets in the early postnatal period leading to adverse metabolic programming later in life [50]. In rodents, dietary manipulations during the early postnatal period cause epigenetic changes that alter growth parameters, lipid synthesis, appetite control, and glucose metabolism in the growing offspring [4]. These processes have also been attributed to GIT microbiota [51].

2.1.5.6 Gut microbiota

The human intestines are inhabited by numerous microorganisms which are influenced by diet, lifestyle and host's genetic make-up [52]. These intestinal microbiome provide several proteins, including enzymes that affect the digestion and absorption of orally ingested food substances [52]. Changes in the composition of gut microbiota may affect the absorption of energy from dietary food and can lead to excess body calorie supply [51]. Thus contributing to the pathogenesis of

various common metabolic disorders including obesity, insulin resistance, hyperglycaemia, type 2 diabetes, non-alcoholic fatty liver disease and cardio-metabolic diseases [51,52].

The gut barrier integrity and permeability is maintained by gut microbiota [53]. This helps prevent deleterious products such as ethanol and endotoxins from entering the portal circulation [53]. However, high-calorie diet (including fructose) is associated with increased bacterial growth and intestinal permeability, due to the increased ability of the microbiome to harvest excess energy from the diet [32]. The gut microbiota digests and ferments the excess energy into short-chain fatty acids thereby making them absorbable by the intestinal epithelium [32,53], and increasing gut acidity. This results in the production of harmful bacterial products, lipopolysaccharides, which activates inflammatory cascades [32]. Additionally, bacteria from the gut microbiota can produce ethanol and endotoxins [53]. These toxic products contribute to the inflammatory processes that can damage the protective intestinal epithelium, leading to the development of NAFLD and metabolic syndrome [53]. Gut microbiota can be affected by poor dietary habits and physical inactivity [54].

2.1.5.7 Poor dietary habits and physical inactivity

Nutrition is the main environmental factor for the development of metabolic syndrome [44]. Diets and nutrients can modify and regulate the functions of proteins in controlling the metabolic processes of the body [42]. Excess nutrition that is associated with a sedentary lifestyle can result in energy intake exceeding energy utilisation, leading to fat accumulation mainly in form of non-esterified fatty acids (NEFA) in susceptible individuals, and these contribute to insulin resistance, oxidative stress and inflammation [2].

Diets high in fat/sugars can promote the activity of the mitochondrial respiratory chain thus, leading to the excess production of mitochondrial ROS [42]. The generated oxidative stress causes a decreased adenosine triphosphate (ATP) production as well as the development of insulin resistance, thereby affecting the overall cellular metabolism leading to the development of metabolic syndrome [2]. Furthermore, diet-induced oxidative stress directly interferes with lipid oxidation resulting in the excessive accumulation of fat in tissues, a condition that interferes with insulin signalling and translocation of the glucose transporter type 4 (Glut-4) in insulin-sensitive tissues, resulting in elevated blood glucose levels and eventual hyperglycaemia [42]. In a situation of sedentary lifestyle, some of the diets that contribute to the development of metabolic disorders include excess dietary fructose [8,55–57].

2.1.6 High-fructose diets in the aetiology of metabolic syndrome

Fructose is a sweet-tasting monosaccharide that is present in sucrose (table sugar), fruits, honey, high fructose corn syrup (HFCS) and sugar-sweetened beverages [58]. Fructose consumption in any of these forms is rapidly increasing globally and has been attributed to the increasing prevalence of metabolic disorders including obesity, type 2 diabetes, dyslipidaemia, NAFLD and cardiovascular disease [57]. Different mechanisms have been proposed to explain the pathogenesis of fructose-induced metabolic derangements, all of which are attributed to the nature in which fructose is metabolised in the tissues [55].

Most of the ingested fructose is metabolised by the liver [57]. It is absorbed into the portal vein from the GIT, after which it is then transported into the hepatocytes by glucose transporter type 2 (GLUT-2) [59], where it is metabolised and converted to fatty acids, triglycerides, and LDL-c which predisposes to obesity, dyslipidaemia, insulin resistance and type 2 diabetes and metabolic syndrome [55]. Unlike glucose metabolism which is regulated by the phosphofructokinase depending on the amount of energy produced and insulin secreted, the metabolism of fructose does not depend on the level of insulin secreted or energy dissipated [55].

In the hepatocytes, fructose is phosphorylated to fructose-1-phosphate by fructokinase, and then to glyceraldehyde and dihydroxyacetone phosphates (triose-phosphates) by an aldolase, with eventual formation of glyceraldehyde-3-phosphate [58]. These metabolic products of fructose are the primary sources of phospho-lipids, triacylglycerol (TAG) and fatty acids. Additionally, fructose metabolism produces a high concentration of lactate, a major source of acetyl-CoA which is converted to TAGs and VLDL [58].

The rapid conversion of fructose to fructose-1-phosphate is believed to be responsible for hepatic ATP depletion, resulting in rapid production of AMP and subsequent conversion of AMP to uric acid [58]. And this is one of the mechanisms by which fructose leads to mitochondrial dysfunction, elevated levels of ROS and oxidative stress [55], that eventually lead to inflammation characterized by elevated pro-inflammatory cytokines, apoptosis and fibrosis [60]. These processes are implicated in the development of fructose-induced endothelial dysfunction, atherosclerosis, hypertension, cardiovascular disease and metabolic syndrome [61].

Fructose impairs PPARα signalling leading to decreased expression of PPARα target genes [61]. This results in mitochondrial dysfunction and a decrease in mitochondrial beta-oxidation of fatty

acids in the liver leading to visceral fat accumulation that contributes to an increase in the whole body mass [57,61].

The sensitivity of insulin can be affected by fructose-induced lipogenesis leading to insulin resistance [62]. The fructose-induced insulin resistance inhibits the insulin-mediated glucose metabolism leading to hyperglycaemia and eventually type 2 diabetes [62]. Adiponectin is metabolically beneficial in that it increases insulin sensitivity, enhances energy metabolism and activates PPARα thereby promoting fatty acid oxidation [39]. The adiponectin signalling may be decreased by high-fructose diets, which may lead to decrease insulin sensitivity and decrease inhibition of lipogenesis thereby predisposing individuals to insulin resistance, visceral obesity and metabolic syndrome [39].

The effects of fructose to increase *de-novo* lipogenesis and inflammation are largely mediated via affecting the expression of certain genes and receptors [61,63]. These genes and receptors are associated with carbohydrate response element-binding protein (ChREBP), Sterol regulatory element-binding protein 1 (SREBP-1), TNF-α and PPARα [63]. These genes and receptors are dysregulated secondary to excess high-fructose diets leading to *de-novo*, hepatic lipid accumulation and inflammation, all of which predisposes to the development of NAFLD [32]. The fructose-induced inflammatory and oxidative processes can generate TNF-α, IL-6 and thiobarbituric reactive substances (TBARS) thereby destroying hepatic cells leading to an increase in circulating liver enzymes including alanine aminotransferase (ALT) [31,64].

In addition to the liver being the major organ for fructose metabolism, some fructose is also metabolised in extrahepatic tissues including the small intestine, kidney, pancreas and testes [65,66]. Thus, sustained excess fructose consumption could lead to the development of obesity-associated pancreatic and cardiovascular dysfunctions [66]. Decreased reproductive performance has been reported in males as a result of excess epididymal fat deposition and reduced testicular weight secondary to chronic high-fructose diet consumption [67].

In the kidney, the metabolism of fructose could damage the renal parenchyma secondary to fructose-induced oxidative stress, and this contributes significantly to the development and progression of nephropathy [65,68]. The fructose-induced nephropathy can lead to the elevation of kidney injury molecule 1 (KIM-1) and tissue malondialdehyde (MDA), which are markers of renal disease [69,70]. Bone mineralisation [71] and red blood cell indices [packed cell volume (PCV),

haemoglobin (Hb), mean corpuscular haemoglobin concentration (MCHC)] [72] have been shown to be adversely affected by excess dietary fructose consumption.

2.1.7 Complications of metabolic syndrome

Long-term metabolic derangements are associated with specific or multiple organ complications (some of which are discussed earlier) that can lead to decreased or limited daily physical activity, thereby affecting economic productivity [73]. These complications are associated with high rates of morbidity and/or mortality, and include but not limited to the following:

2.1.7.1 Type 2 diabetes

Type 2 diabetes is a complication of metabolic syndrome as a result of oxidative and inflammatory processes with accompanying insulin resistance [2,74]. Type 2 diabetes is associated micro- and macro-vascular complications which contribute to the development of renal and cardiovascular diseases [73].

2.1.7.2 Cardiovascular diseases

Micro-vascular damage can result from insulin resistance, leading to endothelial damage, atherosclerosis, vascular resistance and hypertension, which (hypertension) can adversely affect peripheral vascular resistance and stiffness leading to the development of peripheral vascular and structural heart disease [2]. Dyslipidaemia, low adiponectin concentration and the pro-inflammatory cytokines released from the adipose tissue also contribute to the development of atherosclerosis and coronary artery disease, which can lead to the development of ischemic heart disease and stroke [75]. Co-existence of two or more risk factors of metabolic syndrome can increase the rate of mortality from cardiovascular complications [8].

2.1.7.3 NAFLD/NASH

Non-alcoholic steatohepatitis (NASH) is a progressive form of NAFLD secondary to metabolic dysfunction, and can further progress to fibrosis, cirrhosis, and hepatocellular carcinoma [76]. It has been shown that NAFLD can be associated with type 2 diabetes thereby increasing the risk of poor cardiovascular outcomes [28].

2.1.7.4 Chronic kidney disease

Chronic kidney disease and microalbuminuria have been shown to be associated with metabolic syndrome [74]. Nephropathy is the main cause of end-stage renal disease, and is characterized by

glomerular and tubular structural and functional alterations [77], which may adversely affect urinary space, glomerular tuft and renal capsular areas [78,79].

2.1.7.5 Other complications

Other complications arising from metabolic dysfunction include altered red blood cell indices [72], osteoporosis [75] and testicular dysfunction (in males) [67]. These complications are believed to be secondary to inflammation and oxidative stress associated with the pathogenesis of metabolic syndrome [75]. The components of metabolic syndrome and associated complications can be programmed in the early neonatal period of development [80].

2.2 Neonatal programming of metabolic disorders

2.2.1 Developmental Origins of Health and Disease (DOHaD)

The hypothesis on the developmental origins of health and disease (DOHaD) posits that environmental and nutritional influences in critical developmental periods can result in epigenetic changes which can temporarily or permanently alter phenotype physiology and susceptibility or resistance to disease [50,81]. The "single-hit" hypothesis of metabolic programming puts forward that the "single hit" nutritional insult during the early developmental stages leads to metabolic dysfunction immediately or later in life [82]. Whilst the "double-hit" hypothesis of metabolic programming states that the risk of developing metabolic derangements following a perinatal nutritional insult is increased after a second nutritional insult is introduced later in life [83].

Recently, growing evidence has shown that predisposition to metabolic programming involves multiple factors that act together in subjects who are genetically predisposed, a concept described as "multiple hit hypothesis". These factors include nutrition and genetic/epigenetic predisposition [32], and they can affect the offspring's immature satiety centre in the brain which controls appetite. As such, nutrition and weight gain cannot be regulated sufficiently during the neonatal period [80,84].

The early stages of intestinal development involve the formation of the bacterial ecosystem, which can influence the metabolic processes of the host [51]. These early developmental stages of the intestines constitute a "critical window" for programming metabolic health and its metabolic phenotype [51]. Thus, derangements in the intestinal developmental processes secondary to early life dietary manipulation can lead to the development of metabolic disorders in the subsequent developmental stages [51].

High-fructose diets are amongst the dietary nutrients that can have an effect during the neonatal period [85]. Excessive intake of high fructose diets during the perinatal period may serve as a 'first hit' insult that sensitizes the body systems to a 'second hit' or subsequent multiple hit insults [53]. These hits trigger the development of metabolic diseases, including, central obesity, dyslipidaemia, type 2 diabetes, NAFLD and metabolic syndrome in the subsequent developmental periods of life [32,86]. Excessive consumption of dietary fructose and phytochemicals during the neonatal period can lead to alterations in the individual's epigenetic machinery [48].

2.2.2 Epigenetics of neonatal metabolic programming

Genes and gene products can be chemically marked, resulting in information being inherited through gene expression without altering the DNA sequence, a process known as epigenetics [48,49,87,88]. Epigenetic programming may be the underlying mechanism for origins of early life adverse or beneficial metabolic health outcomes in subsequent developmental stages. It (epigenetic programming) can either silence genes entirely or cause the genes to overexpress/under express their protein products [48,49,80,81,89].

Three main epigenetic processes that closely interact have been recognized, and they include DNA methylation, histone modification and non-coding microRNA [48]. These epigenetic processes can occur during neonatal life and persist into adult life [88] and can be influenced by metabolic and environmental factors [48]. Gene expression can be altered by developmental plasticity to produce a suitable phenotype for future environment [87]. The resulting phenotype may match its environment and the organism remains healthy [90]. However, the reverse is the case when there is a mismatch between the phenotype and its environment, and the response to the environmental challenges would not be adequate thereby increasing the risk of developing future diseases [90]. The degree of mismatch can be affected by either poorer or richer environmental conditions during the developmental period, or both [87]. For instance, the degree of mismatch can be increased by the consumption of excessive calorie diets (including fructose) that is associated with physical inactivity, leading to long-term development of metabolic disorders [87].

Gene expression can be potentially mediated and regulated by DNA methylation to bring about susceptibility to or protection against metabolic diseases during the developmental and

differentiation stages of body tissues [87,89]. It has been shown that perinatal dietary manipulation in animals alters gene expression in the developing offspring [87]. For instance, early life nutritional manipulation has been observed to potentiate the expression of PPARα in the liver and heart. This increased PPARα expression is attributed to hypomethylation of their respective promoters [87].

Neonatal nutrition and genetic predisposition can affect the epigenetic mechanisms leading to the development of metabolic diseases including obesity, diabetes and cardiovascular diseases [48]. There are three possible mechanisms of diet-induced epigenetic changes, and they include chromatin machinery modification, ligand activation of nuclear receptor and membrane receptor signalling [48,49]. These epigenetic mechanistic processes are implicated in the regulation of genes involved in metabolic processes including adipogenesis, insulin signalling, glucose homeostasis and inflammation [48].

Epigenetic alterations are reversible and serve as potential therapeutic approaches through silencing or activating morbid genes thereby restoring the normal epigenome [48]. The altered epigenetic patterns can be identified and used as new targets for the development of appropriate diets or drugs, which can prevent or destroy abnormal gene silencing involved [88]. Altered epigenetic mechanisms can lead to the development of metabolic dysfunction not only in adults but also in the paediatric age group [90].

2.3 Metabolic syndrome in the paediatric age group

Childhood is a transitional period of growth and development characterized by significant physiological alterations, which make the assessment of cardio-metabolic status difficult [34]. During this period, the growing organism undergoes numerous physiological modifications that can affect cardio-metabolic characteristics; as such, there is no universally accepted definition of metabolic syndrome in children [34]. In addition, ethnic differences in anthropometric and metabolic parameters have also made it difficult to develop a universally accepted definition of metabolic syndrome [34].

Although several different definitions for assessing metabolic syndrome in children and adolescents were identified since 2003, however, a significant number of these definitions are for the adult classification of metabolic syndrome, particularly National Cholesterol Education Program-Adult Treatment Panel III (NCEP-ATP III) [34,91].

Adult definition of metabolic syndrome cannot be suitable for children because children require the use of threshold specific percentiles for age, sex, and ethnicity to assess cardio-metabolic parameters [34]. Thus considering the socio-demographic characteristics highlighted above, the NCEP-ATP III diagnostic criteria for metabolic syndrome in children and adolescent was modified by substituting the adult cut-offs with the paediatric threshold on specific age and sex percentiles, and ethnicity [34].

There was a unanimous agreement that a universal definition of metabolic syndrome should include four main defining features: dysglycaemia, increased blood pressure, dyslipidaemia, and central obesity [91]. However, to date, there are no uniform cut-off values for the diagnostic criteria in the paediatric age group [91]. Based on these modifications, the most widely used definitions are those of Cook et al. [92] and IDF [93]. Both definitions recognise the presence of four main defining features of metabolic syndrome stated earlier.

In 2007, the IDF developed new clinical diagnostic criteria for paediatric metabolic syndrome, in which significant central obesity (determined by waist circumference) is recognized as a major and essential risk factor, plus two or more risk factors which are determined using adult cut-off values [93] (Table 2.2). Additionally, the IDF proposes that metabolic syndrome should only be assessed in children of 10 years and above, whilst only waist circumference should be used screening children of less than 10 years [34]. Thus, the IDF definition of paediatric metabolic syndrome is considered the most useful clinically.

However, NAFLD, which is also an additional component of metabolic syndrome and maybe a strong cardio-metabolic risk factor, is not considered in the new definition of paediatric metabolic syndrome, despite a reasonable number of obese children and adolescents have already developed NAFLD [91]. Thus, this is a drawback to the current definitions of metabolic syndrome in the paediatric age group.

Table 2.2: The IDF criteria for the diagnosis of metabolic syndrome in children and adolescents

IDF Criteria	**Cut-off values**
Central obesity*	< 10 years: WC ≥ 90th percentile
	10 – 15 years: WC ≥ 90th percentile
	> 15 years (Adult criteria): WC ≥ 94 cm (♂) WC ≥ 80 cm (♀)
Dyslipidaemia	Triglycerides ≥ 1.7 mmol/L
	HDL-c < 1.03 mmol/L (♂) HDL-c < 1.29 mmol/L (♀)
Hypertension	Systolic blood pressure ≥ 130 mmHg or Diastolic blood pressure ≥ 85 mmHg
Fasting glucose	≥ 5.6 mmol/L or diagnosis of type 2 diabetes mellitus

* *= major criteria that must be present, then plus any other two or more criteria; IDF = international diabetes federation; WC = waist circumference; HDL-c = high-density lipoprotein cholesterol; ♂ = males; ♀ = females. Table modified from Zimmet et al.* [93].

The average global prevalence of metabolic syndrome in the paediatric age group is 13.4%, and this prevalence is rapidly increasing in both children and adolescents [34], due to increased consumption of high-calorie diets [94]. It has been shown that childhood metabolic syndrome could predict cardio-metabolic disorders in adulthood. For instance, it has been shown that pre-puberty obesity is invariably associated with a greater risk of developing type 2 diabetes and cardiovascular disease after puberty [91]. Thus, children with greater risk of developing metabolic syndrome and cardio-metabolic risk factors can be identified at the early stage by determining the waist circumference and BMI, since bodyweight status correlates well with cardio-metabolic derangements [34,91]. Consequently, management interventions during the early developmental stages can be potential preventive strategies for the development of full-blown metabolic syndrome in adulthood [94].

2.4 Management of metabolic disorders

The defining criteria for metabolic syndrome in all age groups are generally linked to an unhealthy lifestyle, a risk factor that can be modified [95]. For patients suffering from metabolic

syndrome and in the absence of overt risk factors, lifestyle modification can be used to effectively reverse the syndrome [5]. Thus, the first line management intervention for metabolic syndrome is targeted at lifestyle modification through a multiple approach strategy that includes a healthy diet and physical exercise [95].

2.4.1 Lifestyle modification

Excessive consumption of energy-dense foods associated with a sedentary lifestyle is believed to contribute to the increasing prevalence of metabolic syndrome, type 2 diabetes and cardiovascular diseases, not only in adults but also in children and adolescents [44]. Since decreased intake of excess calorie diets could decrease weight gain, the focus is on decreasing the daily caloric intake and weight loss, and this has a beneficial impact on managing the components of metabolic syndrome including central adiposity, insulin resistance, dyslipidaemia and hyperglycaemia [44]. Consuming low-calorie diets can improve some metabolic derangements even if the rate of weight loss is slow [95].

It has been recognised that high dietary carbohydrate, fatty acids and cholesterol are risk factors for obesity, dyslipidaemia, insulin resistance, NAFLD and cardiovascular disease, and these are the major components of metabolic syndrome [44]. Therefore, diets low in carbohydrate, low in cholesterol and low in saturated fatty acids may improve glucose metabolism, insulin sensitivity and plasma lipid and lipoprotein metabolism [44]. These should be reinforced with increasing exercise to decrease and/or maintain weight. This is an essential approach in managing metabolic syndrome since it has been shown that physical inactivity is associated with increased prevalence of metabolic syndrome, whereas high physical activity is likely to protect against the development of metabolic syndrome [44,95].

Healthy dietary habits and regular exercise are behavioural interventions that can provide short-term benefits and changes in BMI and abdominal circumference, and may improve the risk of developing long-term cardiovascular diseases [95]. However, in severe cases of metabolic dysfunctions with classical risk factors like type 2 diabetes or hypercholesterolaemia, there is a need for conventional pharmacological interventions which should be based on clinical guidelines of a local setting [5].

2.4.2 Conventional pharmacological therapy and surgical interventions

The pharmacological therapies currently available for treating metabolic syndrome are targeted towards specific components or risk factors associated with the syndrome, and the aim is to reduce or prevent the risk of developing long-term complications including cardiovascular disease and type 2 diabetes [96]. Examples of drugs currently in use include fenofibrate (hypolipidaemic), pioglitazone and metformin (anti-hyperglycaemic and insulin sensitizers) [96]. Interestingly, some drugs may have beneficial effects on two or more components of metabolic syndrome [44]. For example, pioglitazone also decreases blood levels of TGs and increases the levels of HDL-c, in addition to its insulin-sensitizing effects [97]. Fenofibrate also reduces inflammation and improves vascular functions [97].

Bariatric surgery may be considered in patients with BMI ≥ 40, or patients with a BMI of 35 with related co-morbidities, as a means of reducing increased body weight and BMI [44]. However, bariatric surgery is not usually recommended for obese children and adolescents unless lifestyle modification and conventional pharmacologic interventions are not effective in attenuating the increased body weight and BMI [91].

Whilst the medical and surgical interventions are used in managing the components of metabolic syndrome, however, they have limited effectiveness and associated with adverse side effects [96]. Additionally, pharmacological and surgical interventions are not usually recommended for children and adolescents as stated earlier [91]. Thus, phytomedicinal therapy is currently being explored for the prevention and management of metabolic disorders.

2.4.3 Phytomedicinal therapy

Medicinal plants are globally recognised as important components of healthcare systems [98], as they are widely used in the treatment and prevention of various health conditions including diet-induced metabolic disorders [99]. Majority of the global populations rely on medicinal plants for their healthcare needs [98], since these medicinal plants are usually cheap and readily available [100]. The WHO has encouraged the exploration, scientific validation and standardisation of herbal medicines worldwide [98,101], and this has opened windows of research for alternative therapies using natural plants including ginger [100].

Ginger is derived from the rhizome of the ginger (*Zingiber officinale* Roscoe) plant (Figure 2.1) [102]. The plant belongs to the family *Zingiberaceae* with origins in South-East Asia, however this perennial plant is currently widely cultivated in the remaining parts of the world [103]. Ginger has medicinal and nutritional benefits making it an important spice used for flavouring and therapeutic purposes worldwide [100].



Figure 2.1: Uprooted and washed Ginger plants showing the prominent rhizomes (Source: https://images.app.goo.gl/qNGRjHgHzfsx9RHC8)

Ginger consists of different organic phytochemicals [102]. Studies have shown that the health beneficial effects of ginger are due to these active phytochemicals [100,102]. One of such phytochemicals is zingerone, which was the target phytochemical in this study.

2.5 Zingerone

Zingerone (Vanillyl acetone) is a bioactive chemical compound present in the plant ginger (*Zingiber officinale*) [69]. It is estimated that about 9.25% of zingerone is present in ginger, and is widely used as a flavouring agent that provides spicy aroma in food and drinks [102]. Zingerone

belongs to the family of Methoxyphenol with a basic phenolic ring, in which a methoxy group is attached to benzene ring [102]. Thus, zingerone is a phenolic ketone with a chemical formula [4-(4-hydroxy-3-methoxyphenyl)-2-butanone] (Figure 2.2), and this makes it a strong antioxidant as it possesses a hydroxyl group structurally [104].

Zingerone is sparingly soluble in water [41] and has a density that ranges between 1.138-1.139 at 25°C [105]. After oral ingestion, zingerone is best and passively absorbed in the small intestine and colon via first order kinetics [105], and has oral absolute bioavailability of 72.2% [106]. Within 15 minutes of oral administration, zingerone is entirely distributed in the body tissues especially the liver, stomach, intestines, pancreas, kidney and heart [105]. Zingerone is mainly excreted in urine (40%) and faeces (50%) as homovanillic acid and conjugates of glucuronide and/or sulfate after 24 hours of oral administration [102,105].

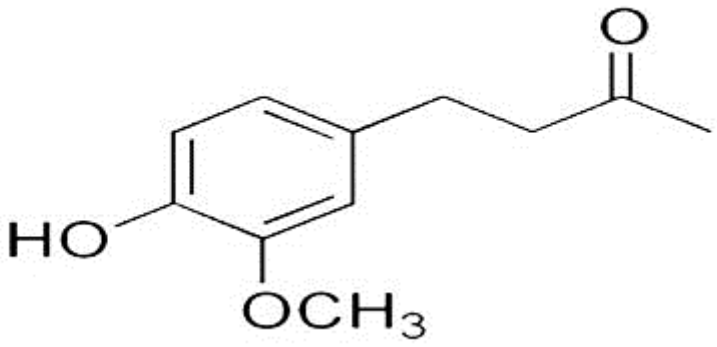

Figure 2.2: Zingerone's chemical structure [4-(4-hydroxy-3-methoxyphenyl)-2- butanone] [107].

Studies have attributed a wide range of pharmacological properties to zingerone, including lipolytic, hypoglycaemic anti-oxidant, anti-inflammatory properties to mention but a few [99,103,104].

2.5.1 Zingerone as a hypolipidaemic and anti-obesity agent

Hypertrophy and hyperplasia of adipose tissue secondary to altered lipid metabolism are the hallmark of obesity [36], and these translate to increased circulating TGs, and LDL-c, and decreased levels of HDL-c with eventual predisposition to increased body weight and metabolic syndrome [2].

A study conducted by Narayanan and Jesudoss [108] showed an increase in body weight and BMI, as well as derangement in the lipid profile where the levels of TG, LDL-c and total cholesterol were raised and HDL-c was reduced following consumption of fructose enriched diets (40% fructose diet plus 20% solution) by adolescent male Wistar rats for eight weeks. Zingerone administration at 100 mg/kg body weight significantly decreased the body weight, BMI, levels of TG, LDL-c and total cholesterol and elevated the levels of HDL-c of the rats. Similar beneficial effects of zingerone (on lipid profile) were observed by Jothi et al. [101] and Ahmad et al. [103] on STZ-induced and alloxan-induced diabetic Wistar rats respectively. Additionally, zingerone administered to adolescent male Wistar rats at 6 mg/kg body weight for 2 weeks attenuated hyperlipidaemia (increased TGs, LDL-c and decreased HDL-c levels) in isoproterenol-induced myocardial infarcted rats [106].

The mechanism of action related to hypolipidaemic effects of zingerone was attributed to its (zingerone) ability to induce norepinephrine-mediated lipolysis in the adipose tissue of the study animals. Stimulation of norepinephrine-induced lipolysis by zingerone is attributed to the translocation of hormone-sensitive lipase from the cytosol to adipocytes thereby preventing fat accumulation [99,104,108].

2.5.2 Zingerone as a hypoglycaemic and anti-diabetic agent

Hyperglycaemia is primarily a consequence of insulin resistance secondary to beta cell dysfunction [2]. These pathologic effects are attributed to the inflammatory and oxidative processes that generate cytokines and ROS respectively; both of them affect beta cell function and insulin sensitivity leading to type 2 diabetes [2,35]. Zingerone has been shown to correct hyperglycaemia and prevent the development of type 2 diabetes in rats.

In a study conducted by Jothi et al. [101], 40 mg/kg streptozotocin (STZ) was used to induce diabetes in adolescent male Wistar rats, which had led to the development of hyperglycaemia. Zingerone administered at 10 mg/kg for 30 days prevented the elevated blood glucose level. Fructose-induced raised blood glucose level was also attenuated following zingerone administration at 100 mg/kg body weight to adolescent male Wistar rats for eight weeks [108]. Ahmad et al. [103] observed that treatment with zingerone (50 and 100 mg/kg) for 3 weeks protected adolescent male Wistar rats against alloxan-induced diabetes by improving insulin level and sensitivity. Furthermore, both fasting blood glucose and homeostatic model assessment

of insulin resistance (HOMA-IR) index were reduced in STZ-induced diabetic adult male rats after treatment with zingerone (50 and 100 mg/kg) for 16 weeks. All these reports showed that zingerone possesses anti-hyperglycaemic properties [69].

The hypoglycaemic and insulin-sensitizing effects of zingerone have been attributed to the ability of zingerone to regenerate pancreatic islets and stimulate insulin release thereby protecting against diabetes [103,109]. This protection of diabetes by zingerone occurs via regulation of oxidative stress and inhibition of inflammatory processes [103].

2.5.3 Zingerone as an anti-oxidant and anti-inflammatory agent

Oxidative stress is a condition in which body cells are exposed to high levels of oxygen or ROS, and this can affect adipocytes leading to oxidative damage to lipids known as lipid peroxidation [41]. As a result, lipid peroxidation products are generated directly (such as MDA) or indirectly (such as TBARS) [105]. Whilst inflammation is the body's response to harmful stimulus leading to production of inflammatory cytokines including TNF and IL-6, which can be harmful to the body tissues [105], and are invariably associated with oxidative stress [69].

Metabolic diseases including obesity, type 2 diabetes, NAFLD and diabetic nephropathy are usually associated with oxidative stress and inflammatory processes, in which there is increased production of ROS and upregulation of pro-inflammatory cytokines [2,35,69,103]. Many studies have documented the effectiveness of zingerone in attenuating oxidative stress by decreasing the levels of MDA and TBARS, and downregulating cytokines including TNF-α and IL-6 [69,103,105,110].

A study was conducted in which alloxan-induced diabetic male Wistar rats were observed to have elevated levels of MDA, TNF-α and IL-6, these were attenuated following three weeks treatment with zingerone at 50 and 100 mg/kg body weight [103]. Similar observation was made by Rehman et al. [69] following 16 week treatment with similar doses of zingerone in STZ-induced adult male diabetic rats. It has also been shown that zingerone possesses free radicals scavenging ability in food radiolysis, thereby attenuating lipid peroxidation of food substances [102,104].

The anti-inflammatory and anti-oxidative effects of zingerone are attributed to its multiple actions, which include hydroxyl group free radical scavenging on aromatic ring and presence of double bond between C-4 and C-3 of zingerone. Facilitation of proton release by the methoxy group from the phenolic hydroxyl group, downregulation of inflammatory processes and increasing the activity of hepatic PPARα, one of the key regulators of various metabolic

pathways as well as inflammation are additional mechanisms of action [104,108]. The anti-oxidant and anti-inflammatory activities of zingerone provide an opportunity for the treatment and prevention of diseases associated with lipid peroxidation and inflammation, including hepatic diseases [104].

2.5.4 Zingerone as a hepatoprotective agent

The liver plays a significant metabolic role in the body, and participates in the inflammatory pathways to get rid of some deleterious stimuli [105]. Hepatic inflammatory process can be caused by exogenous substances such as environmental toxins, as well as endogenous substances such as ROS [105]. The main factors implicated in hepatic injury include hepatic fat accumulation, lipotoxicity secondary to oxidative stress with elevated oxidant markers (MDA and TBARS), and release of inflammatory cytokines (TNF-α and IL-6) [111]. Additionally, hepatic inflammation could also be caused by increased apoptosis [111]. These deleterious processes ultimately lead to hepatic tissue damage, NAFLD and eventually NASH which is characterised by inflammatory cell aggregates and fibrotic changes in the hepatocytes [111].

Zingerone administration at 100 mg/kg to male Wistar rats for eight weeks attenuated the hepatic lipid accumulation and hepatic enzyme markers secondary to consumption of fructose-enriched diet, thereby protecting against hepatic tissue damage and development of NAFLD/NASH [108]. Mani et al. [107] observed reduced alterations in the liver enzymes and hepatic damage after a four-week supplementation with 40 mg/kg zingerone to male albino Wistar rats exposed to ethanol-induced liver toxicity. In another study it was reported that treatment with zingerone at 50 mg/kg for three weeks attenuated the alloxan-induced elevated serum levels of hepatic enzymes in alloxan-induced diabetic Wistar rats [103]. Similar effect of zingerone was observed by Julietpoornamathy and Parameswari [112] on hepatic enzymes.

Narayanan and Jesudoss [108] and Zaki et al. [111] reported decreases in vesicular steatoses, inflammation and fibrosis (features of NAFLD and NASH) in the liver histological sections of fructose-fed rats following administration of zingerone. Similar beneficial effect of zingerone was made by Mani et al. [107] on the liver histological sections of rats exposed to alcohol-induced hepatic damage.

The hepato-protective effects of zingerone have been attributed to its ability to attenuate hepatic steatosis, reduce oxidative stress as well as lipid peroxidation [106]. Additionally, prevention of

DNA damage mediated by ROS, stabilisation of hepatocytes membranes thereby preventing leakage of hepatic damage markers (liver enzymes including ALT) to the extracellular fluid) and downregulation inflammatory cytokines (including IL-6 and TNF-α) of hepatic inflammation also occur [110,112,113].

2.5.5 Zingerone as a nephroprotective agent

Diabetic nephropathy is one of the major microvascular complications associated with type 2 diabetes and metabolic syndrome [103], and is linked with oxidative stress, inflammatory process, renal function alterations, renal lesions and fibrosis [69]. In diabetic kidney disease, excess production of ROS is due to either direct effect of hyperglycaemia or via cytokine release [69].

Following a four-week administration of zingerone (40 mg/kg) to the male Wistar rats that were exposed to alcohol-induced kidney damage, the elevated levels of renal function markers including uric acid were returned to normal, suggesting that zingerone was able to preserve the renal physiology against ethanol-induced renal damage [107]. Additionally, the authors of the same study observed damaged renal glomeruli and tubules on histology, the pathology that was reduced by administration of zingerone thereby restoring the normal cellular structure of the kidney. In another study conducted by Rehman et al. [69], STZ-induced diabetes resulted into increase in kidney levels of ROS and MDA, serum levels of KIM-1, creatinine and BUN in the study rats. Treatment of the study rats with 100 mg/kg of zingerone for 16 weeks decreased the kidney levels of ROS and MDA, as well as the serum levels of KIM-1, creatinine and BUN were restored back to physiological values.

The nephroprotective property of zingerone is attributed to its anti-hyperglycaemic, anti-oxidative and anti-inflammatory effects [69,107].

2.5.6 Other pharmacological properties of zingerone

Other pharmacological properties of zingerone include those beneficial effects reported on the heart, GIT, bones, microbial infections, chemotherapy and radiolysis-induced oxidative damage. Overweight, obesity, type 2 diabetes and atherogenic dyslipidaemia are components of metabolic syndrome that are strongly associated with cardiovascular diseases [27]. The pathogenesis of these diseases has been linked to oxidative and inflammatory processes [2]. Based on its anti-oxidative, anti-inflammatory and hypolipidaemic effects, zingerone administered at 6 mg/kg to male albino

Wistar rats for 2 weeks protected the hearts of rats against the isoproterenol-induced cardiac hypertrophy and myocardial infarction [106]. Soliman et al. [114] documented a cardioprotective effect of zingerone (25 mg/kg) against cisplatin-induced oxidative stress and inflammation in adult male rats within 3 weeks of administration.

The beneficial therapeutic effects of zingerone on gastro-intestinal diseases have been demonstrated by numerous studies, and these therapeutic effects are also attributed to the anti-inflammatory and anti-oxidant effects of zingerone [115]. For example, the number and size of ethanol-induced gastric ulcers were decreased by administration of zingerone at doses 50 mg/kg to the study rats. This histological effect of zingerone was attributed to decreased levels of tissue MDA and TBARS, which are the direct and indirect markers of lipid peroxidation respectively in the rat stomach [115].

Zingerone has also been shown to have beneficial therapeutic effects on bones, in which treatment with zingerone stimulated osteogenesis via cellular differentiation of osteoblast in mesenchymal stem cells of mouse. Thus, bone and bone-related disorders would be treated using the osteogenic potential of zingerone [116]. Zingerone has been shown to exhibit antimicrobial activity on different Gram-positive and Gram-negative bacteria [104]. In the third world countries, the leading cause of death is *Escherichia coli* induced diarrhoea. It was reported that zingerone protected against *E. coli* induced diarrhoea by inhibiting the bacterial enterotoxins and colonic movement in rats [102,104]. Furthermore, it has been demonstrated that zingerone inhibited biofilm formation of *Pseudomonas aeruginosa*, a Gram-negative nosocomial bacteria [104].

The potential therapeutic effects of zingerone in the treatment of cancer has been explored.

It has been reported that zingerone supplementation in dimethyl hydrazine treated rats significantly decreased the incidence of tumours [102]. Zingerone also demonstrated promising results in the treatment of colon cancer [104]. The chemotherapeutic effects of zingerone have been linked to its ability to inhibit lipid peroxidation, increase enzymatic and non-enzymatic anti-oxidants' expression, destroy tumour cells' membranes and stimulate autophagy-associated cell death [102,104]. Cytotoxic chemotherapy associated nausea and vomiting, as well as postoperative nausea and vomiting can be prevented with the use of zingerone [102]. This is due to the belief that zingerone non-competitively antagonises serotonin receptors (visceral afferent neurons) in the central and peripheral nervous systems [102].

Patients are exposed to different types of rays during cancer radiation therapy, in addition to diagnostic purposes for variety of respiratory, cardiac, orthopaedic and gynaecological diseases [102]. These rays include X-rays, gamma rays and infrared rays, and can lead to oxidative stress and generation of ROS which damage the exposed organs. Zingerone has been shown to neutralize free radicals generated by radiation-induced oxidative stress [102].

2.5.7 Zingerone safety and toxicity

Despite zingerone showing potential as a medicinal phytochemical, there is limited research and information on its safety and toxicity, and this is a limitation that is faced by most plant-derived medicines [117]. However, the safety of zingerone for oral consumption has been reported by Rehman et al. [69], who considered an established lethal dose (LD_{50}) of 2580 mg/kg body weight of zingerone to be safe and non-toxic in rats. Jesudoss et al. [105] reported a median LD_{50} of 657.55 mg/kg body weight of zingerone in mice after oral administration. In humans, the effective and safe dose of zingerone is reported to be 3 g in adults and 50 mg/kg body weight in children [118]. All these observations are pointers towards the safety of zingerone for oral consumption.

2.6 Influence of dietary nutrients and phytomedicines on the gastro-intestinal tract

Rapid development of the rat's gut occurs during suckling and the immediate weaning period, stages at which dietary manipulation can affect the morphometry of the gut [119]. The expression of certain glucose transporters (GLUT-5, a highly specific fructose transporter, has been shown to be upregulated in the gut following oral ingestion of fructose during the early developmental stages [85]. This can alter the structural and functional integrity of the GIT thereby predisposing the growing organism to the long-term development of metabolic disorders [120,121].

GIT can also be affected by orally ingested phytochemicals. Hyperplastic growth of the intestines and pancreas, as well as disruption of the intestinal brush border have been observed in rats following oral ingestion of phytohaemagglutinin, a lectin from red kidney beans (*Phaseolus vulgaris*) [122–124]. However, ursolic acid [125] and oleanolic acid [126] demonstrated protective effects on the intestinal mucosal barrier and liver respectively.

The next chapter is a published manuscript, and it describes the potential of neonatal orally administered zingerone to programme for protection against long-term development of metabolic dysfunction induced by oral consumption of high-fructose diet in male and female Sprague Dawley rats.

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CHAPTER THREE - Published manuscript 1

Neonatal zingerone protects against the development of high-fructose diet-induced metabolic syndrome in adult Sprague Dawley rats

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(2020)

Journal of Developmental Origins of Health and Disease, pp 1-9.

doi: 10.1017/ S2040174420000525

3.1 Abstract

During the early postnatal period, dietary manipulations can alter the developmental trajectory of the growing offspring, causing beneficial or adverse health outcomes later in adult life. We investigated the potential preventive effects of neonatal zingerone intake on the development of fructose-induced metabolic derangements in rats.

Four-day old male and female Sprague Dawley rat pups (n = 79) were randomly grouped and administered: 10 ml/kg body weight (bwt) of distilled water (W), 10 ml/kg bwt 20% fructose solution (FS), 10 ml/kg bwt fructose solution + 40 mg/kg bwt of zingerone in distilled water (ZF), or 40 mg/kg bwt of zingerone in distilled water (ZW) pre-weaning. After weaning, W and ZW continued on unlimited tap water while FS and ZF continued on unlimited fructose solution for 10 weeks. Body mass and food and fluid intake were evaluated, plasma was collected for metabolic assays and visceral fat was quantified.

Food intake was decreased, fructose and overall caloric intake were increased due to fructose feeding in both sexes ($P < 0.05$). When compared with the controls, the high-fructose diet significantly raised the terminal body masses of females ($P < 0.0001$), concentrations of triglycerides, total cholesterol, LDL-c, TG:HDL-c ratio and visceral fat mass relative to bwt in both sexes ($P < 0.05$). Zingerone prevented ($P < 0.05$) the fructose-induced increase in body mass (females) and hypercholesterolemia (both sexes). Levels of HDL-c, glycaemic parameters and adiponectin were not affected by the interventions ($P > 0.05$). Sex-related differences were observed in food, fluid and caloric intake, terminal mass, cholesterol subtypes and visceral fat percentage ($P < 0.05$).

Zingerone could be used strategically in the neonatal phase as a prophylatic management of high-fructose diet-induced metabolic syndrome.

Keywords: Fructose; metabolic dysfunction; zingerone; neonatal; dyslipidaemia; obesity

3.2 Introduction

The early postnatal period of life is a dynamic developmental period where dietary and environmental interventions can affect an individual's phenotypic physiology, leading to beneficial or adverse health outcomes in subsequent stages of life [1]. Nutrients and phytochemicals can interact with the epigenetic machinery of the individual during the neonatal

period leading to epigenetic changes [2]. These epigenetic changes are associated with dysregulation of gene expression that underlies the developmental programming of adverse health outcomes in adulthood [3]. The diet-induced epigenetic changes occurring during the neonatal period can also affect the individual's immature brain [3].

During the early postnatal period, the differentiating satiety centre in the brain is influenced by intrinsic and extrinsic factors that affect appetite, and consequently, nutrition and weight gain cannot be adequately regulated [4]. The dietary nutrients that can have an effect during the neonatal period include high-fructose diets [5]. Excess dietary fructose consumption during childhood leads to the development of cardio-metabolic disorders including central obesity, dyslipidaemia, type-II-diabetes and coronary heart disease in the subsequent developmental periods of life [6]. The aforementioned are amongst the components of metabolic syndrome [7].

Over one billion people in the world are affected by metabolic syndrome, with females having a higher prevalence (51.7-53%) than males (43%), while children account for 3-4% [8,9]. This high prevalence of metabolic syndrome is associated with an increase in fructose consumption among other factors [10].

Most of the ingested fructose is transported to the liver where it is metabolised leading to visceral fat deposition, dyslipidaemia, obesity, excessive weight gain, insulin resistance and type-II-diabetes [11]. Whilst glucose metabolism is regulated at the level of phosphofructokinase, by the amount of adenosine triphosphate (ATP) generated and insulin secreted, fructose metabolism is independent of these two factors [11]. This results in fructose being converted to fatty acids, triglycerides and low-density lipoprotein cholesterol (LDL-c) which predisposes to the pathogenesis of obesity and metabolic syndrome [11]. Fructose also suppresses hepatic fatty acid oxidation and high-density lipoprotein cholesterol secretion leading to visceral adiposity and an increase in the overall body mass [12].

Fructose-rich diets affect insulin sensitivity via lipogenesis leading to insulin resistance and eventually type-II-diabetes [13]. Adiponectin, an anti-inflammatory adipokine, enhances insulin sensitivity, energy metabolism and fatty acid oxidation by activating peroxisome proliferator-activated receptor alpha (PPARα) [14]. Fructose decreases adiponectin signalling leading to hypoadiponectinaemia, which may affect the extent to which adiponectin inhibits lipogenesis thereby predisposing individuals to visceral obesity and metabolic dysfunction in later life [14].

Management of metabolic disorders remains a challenge globally due to the expense of synthetic conventional drugs and poor healthcare facilities, especially in developing countries, making the drugs inaccessible to most [15]. The currently used drugs also tend to be monotherapeutic, targeting specific components of the metabolic syndrome and hence patients must take several formulations simultaneously and chronically to treat the conditions, resulting in adverse side effects, drug reactions, non compliance and increased costs [16,17]. Given these challenges, there is an increasing interest in the strategic use of medicinal plants and naturally derived phytochemicals with a broad spectrum of biological action [17]. Previous studies have demonstrated positive health outcomes with the strategic use, during the neonatal period, of phytochemicals including S-allyl cysteine [16], oleanolic acid [18] and ursolic acid [19] as protection against metabolic dysfunction.

Zingerone (Vanillyl acetone) is a bioactive ketone found in ginger (*Zingiber officinale*) and is used as a flavouring agent in the food industry [20]. It has demonstrated antiobesity (fructose-induced), hypolipidaemic (fructose-induced) and antidiabetic (alloxan and streptozotocin-induced) properties in adult male rats [21–25]. The effects of zingerone on the liver include increase in PPAR activities and fatty acid oxidation that attenuate lipogenesis and preventing hepatic damage via inhibition of free radicals generation, downregulating inflammatory cytokines [25,26] and decreasing the activity of ALT [26,27] (our target for studies). The biological properties of zingerone make it a possible candidate that could be administered during the neonatal period to protect against long-term adverse metabolic outcomes secondary to high-fructose diets.

Metabolic studies were previously mostly conducted on adult males [10,24,28,29]. However, components of metabolic syndrome are also observed in growing children, with sexually dimorphic features between males and females [3]. We thus explored the potential of neonatal orally administered zingerone to programme for long-term protection against the adverse metabolic outcomes secondary to a high-fructose diet in male and female growing rats.

3.3 Methods

3.3.1 Study setting

The study was undertaken at the Central Animal Service of the University of Witwatersrand, Johannesburg, South Africa, following approval by the Animal Research Ethics Committee of the University of Witwatersrand (Reference No: 2017/010/71/B). The study was conducted according to internationally accepted principles for laboratory animal use and care as stipulated in South African National Standard (SANS 10386:2008) and Animals Protection Act, 1962: Act No. 71. The manuscript was compiled in alignment with the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines.

3.3.2 Animal housing

The experiment used 4-day-old male and female rat pups and consisted of a pre-weaning stage (postnatal day 8-21) and a post-weaning stage (postnatal day 22-91). The pre-weaning stage involved housing of the pups in each litter with their dams, respectively, in Perspex cages that were lined with wood shavings. In the post-weaning stage, the pups were housed singly in Perspex cages. In both stages, the room of the animals was kept at a temperature range of 24-26°C with a 12-h dark and light cycles, with lights on from 7:00 h to 19:00 h for all the stages of the experiment.

3.3.3 Study design

A total of 79, 4-day-old suckling male and female Sprague-Dawley rat pups weighing 9.7 ± 1.1 g were received from seven nulliparous dams, each with a litter consisting of 8-12 pups. The sample size ranged between 8 and 12 rats per group, and was calculated based on a previous study conducted in our research Laboratory [16] using the following formula [30]:

$$\text{Sample size} = 2\,SD^2\,(1.96 + 0.842)^2/d^2$$

where SD = standard deviation from previous study; 1.96 = type 1 error of 5%; 0.842 = at 80% power; d = difference between mean values.

The pups were acclimatised to handling and the environment for 4 days, and then randomly allocated to four treatment groups: Group I which served as the negative control (W) [gavaged with 10 ml/kg body weight (bwt) of distilled water before weaning and then provided unlimited access to plain tap water post-weaning, n = 20; Males (M) = 9, Females (F) = 11]; Group II (FS) received fructose only (gavaged with 10 ml/kg bwt of a 20% fructose solution before weaning and then provided unlimited

access to a 20% fructose solution to drink post-weaning, n = 20; M = 9, F = 11) to induce metabolic dysfunction; Group III was administered zingerone (40 mg/kg bwt) neonatally to programme for protection against long-term high-fructose consumption, received 20% fructose solution (10 ml/kg bwt) neonatally and then unlimited access to 20% fructose solution to drink post-weaning (ZF, n = 21; M = 9, F = 12); Group IV administered with zingerone only (40 mg/kg bwt of zingerone dissolved in distilled water before weaning and given unlimited access to plain tap water to drink post-weaning (ZW, n = 18, M = 8, F = 10) to assess the effects of zingerone alone on metabolic health of the animals. In addition, all the pups nursed freely from their respective dams during the pre-weaning stage and later had free access to standard rat chow [Labchef Rodent Breeder, Nutritionalhub (Pty) Ltd, Stellenbosch 7602, South Africa] during the post-weaning stage.

Fructose (Natures Choice, South Africa) was made as a 20% solution by diluting 200 g of fructose in one litre of water [29]. Food grade zingerone (W312401-1KG) was purchased from Sigma-Aldrich (USA).

3.3.4 Measurements

3.3.4.1 Food, fluid and calorie intake

The food and fluid intake by each rat was determined weekly in the last 4 weeks of intervention. At the end of each week, the remaining amounts of food in the cage were subtracted from the total amounts supplied at the beginning of the week. The fluid was provided in freshly prepared predetermined amounts such that there was a constant supply. The remaining fluid was then subtracted from the total supplied at the end of each week. The weekly food and fluid consumption were computed as a percentage of body mass as g/100 g and ml/100 g, respectively. The total weekly calorie intake was determined by multiplying the amount of food and fructose consumed each week by their respective reference calorie values, and the two were then added together.

3.3.4.2 Body mass

The rats were weighed daily during the pre-weaning stage, and then twice a week during the post-weaning stage by individually placing the rats in a pre-weighed cage on an electronic balance (Snowrex Electronic Scale, Clover Scales, Johannesburg).

3.3.4.3 Terminal procedures

On postnatal day 90, the rats were fasted from solid feed but provided plain drinking water overnight for 12 h. Terminal body mass was then determined (on postnatal day 91) as stated earlier. In order to quantify fasting blood glucose, a drop of blood was taken after a pinprick on the tail vein. The fasting concentration of glucose in the blood was quantified using a glucose meter (Contour Plus Bayer Health Care, Diabetes Care, Isando, South Africa) according to the manufacturer's instructions. The rats were then euthanised with sodium pentobarbitone (Centaur Laboratories, Johannesburg, South Africa) at 150 mg/kg body weight intraperitoneally,which is considered as appropriate and acceptable euthanasia in rats [31].

A ventral midline incision was made on the thorax and abdomen, and intra-cardiac blood was drawn into syringes via attached needles. The blood samples were then transferred into heparinised tubes (BD Vacutainer, Plymouth, UK) and centrifuged (Rotofix 32A, Hettich Zentrifugen, Germany) at 3700 g for 15 min at 20 °C. The plasma was harvested and stored at -20 °C for later assays. Visceral fat (surrounding the liver, kidneys, pancreas, stomach, small and large intestines) was carefully removed and weighed on a Presica 310M digital scale (Precision Instruments, Johannesburg, South Africa).

3.3.4.4 Blood parameters

3.3.4.4.1 Lipid profile

The stored plasma samples were thawed and triglycerides (TGs), total cholesterol (TC), LDL-c and high-density lipoprotein cholesterol (HDL-c) concentrations were determined using specific enzyme-based assay kits (Elabscience Biotechnology Co., Ltd, Wuhan, Hubei, China).

3.3.4.4.2 Adiponectin, insulin and Homeostasis Model of Assessment

Fasting plasma insulin and adiponectin were determined using rat-specific insulin and adiponectin Enzyme-Linked Immuno-Sorbent Assay kits (Elabscience Biotechnology Co., Ltd, Wuhan, Hubei, China). The insulin resistance index was computed according to the Homeostasis Model of Assessment (HOMA-IR) [32].

$$\text{HOMA-IR} = [\text{fasting plasma insulin } (\mu U/ml) \times \text{fasting glucose } (mmol/l)]/22.5$$

3.3.5 Statistical analysis

GraphPad Prism 5 software (Graph-Pad Software Inc., San Diego, USA) was used to analyse the data from all the animals. Data were expressed as mean ± standard deviation. A one-way analysis of variance [(ANOVA), parametric] was used to analyse multiple-group data on food, fluid and calorie intake, blood parameters and visceral fat, while repeated-measures ANOVA was used to analyse within-group data on weekly body mass, food and fluid intake. The multiple-comparisons Tukey *post hoc* test was used to compare the means. A two-way ANOVA was used to compare the effect of variation in treatment, sex and interaction, and statistical significance was considered when $P < 0.05$.

3.4 Results

3.4.1 Food, fluid and calorie intake in adulthood

The weekly food, fluid and calorie intake of the rats in the last 4 weeks of the intervention period is shown in Supplementary Tables S1*A*, S1*B* and S1*C*, respectively. Rats that received fructose with or without zingerone (FS; ZF) had significantly [$P = 0.0005$ (Males); $P < 0.0001$ (Females)] lower food intake compared to the negative controls (W) and the group administered zingerone only neonatally (ZW; Supplementary Table S1*A*). There was no difference [$P = 0.7618$ (Males); $P = 0.1083$ (Females)] in the food intake between the negative controls (W) and the group that received zingerone only (ZW), as well as between the fructose only group (FS) and the group that had fructose and zingerone (ZF) [$P = 0.6511$ (Males); $P = 0.5234$ (Females); Supplementary Table S1*A*].

Rats that received fructose with or without zingerone (FS; ZF) had significantly [$P = 0.0005$ (Males); $P < 0.0001$ (Females)] higher fluid intake compared to the controls (W) and the group that had zingerone only (ZW; Supplementary Table S1*B*). There were no differences [$P = 0.4529$ (Males); $P = 0.3105$ (Females)] in the fluid intake between the control (W) and zingerone only (ZW) groups, as well as between the fructose only group (FS) and the group administered a combination of fructose and zingerone (ZF) [$P = 0.3563$ (Males); $P = 0.1350$ (Females); Supplementary Table S1*B*).

Rats that received fructose with or without zingerone (FS; ZF) had significantly [$P = 0.0003$ (Males); $P < 0.0001$ (Females)] higher calorie intake compared to the controls (W) and the group that had zingerone only (ZW; Supplementary Table S1*C*). There were no differences [$P = 0.2802$ (Males); $P = 0.1113$ (Females)] in the calorie intake between the control (W) and zingerone only

(ZW) groups, as well as between the fructose only group (FS) and the group administered a combination of fructose and zingerone (ZF) [$P = 0.8165$ (Males); $P = 0.9342$ (Females); Supplementary Table S1*C*].

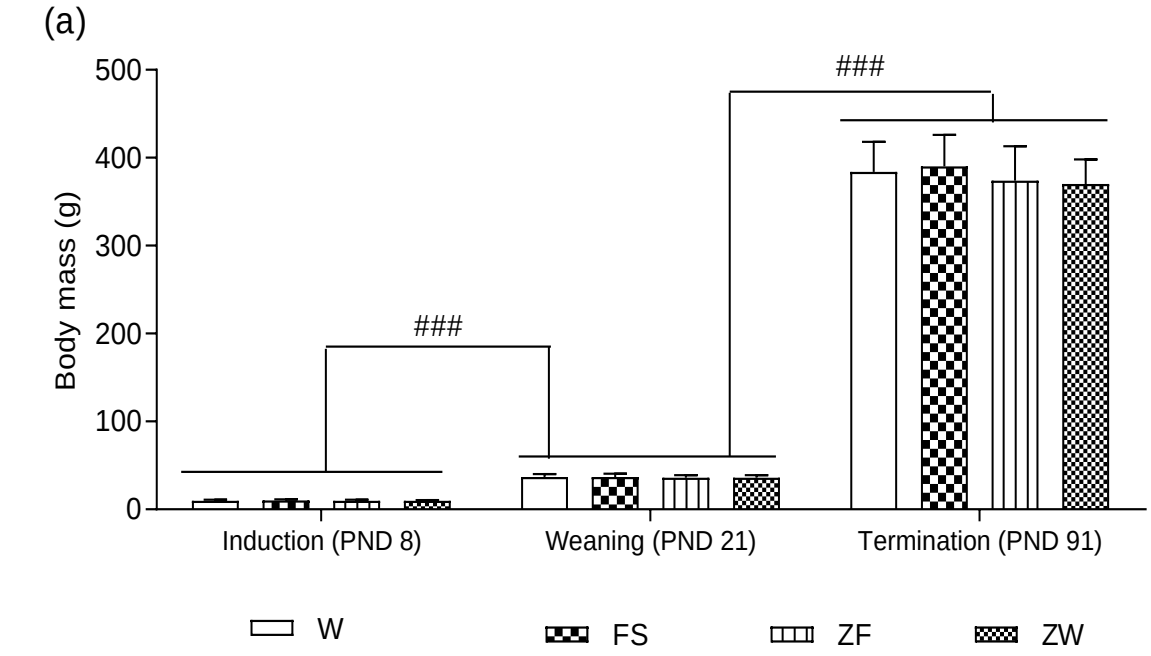
However, female rats had significantly higher food [$P = 0.0002$ (sex effect), $P < 0.0001$ (treatment effect), $P = 0.0028$ (interaction effect)], fluid [$P < 0.0001$ (sex, treatment and interaction effects)] and calorie [$P < 0.0001$ (sex and treatment effects), $P = 0.0015$ (interaction effect)] intake in comparison to their male counterparts.

3.4.2 Body mass

The induction, weaning and terminal body masses of the study rats are represented in Figure 3.1. All the rats grew significantly ($P < 0.0001$) from induction to weaning as well as from weaning to termination. In males, there were no differences in the induction ($P = 0.5976$), weaning ($P = 0.9658$) and terminal ($P = 0.6214$) body masses of the rats across treatment groups (Figure 3.1a).

In females, there were no differences in the induction ($P = 0.3127$) and weaning ($P = 0.1456$) body masses of the rats across treatment groups (Figure 3.1b). However, the high-fructose diet significantly ($P < 0.0001$; FS vs other treatments) increased the terminal body mass of the female rats. Thus, neonatal administration of zingerone prevented the high-fructose diet-induced increase in terminal body mass in female rats. The body weight data showing the longitudinal weight curves at time points in which changes in body weight occurred in male and female rats are shown in Supplementary Figure S1*A* and *B*, respectively.

Despite no difference in the induction [$P = 0.1931$ (sex effect), $P = 0.4517$ (treatment effect), $P = 0.7884$ (interaction effect)] and weaning [$P = 0.0640$ (sex effect), $P = 0.1933$ (treatment effect), $P = 0.8121$ (interaction effect)] body masses between male and female rats, the male rats had significantly [$P < 0.0001$ (sex effect), $P = 0.0310$ (treatment effect), $P = 0.7537$ (interaction effect) higher terminal body masses than females (Figure 3.1c).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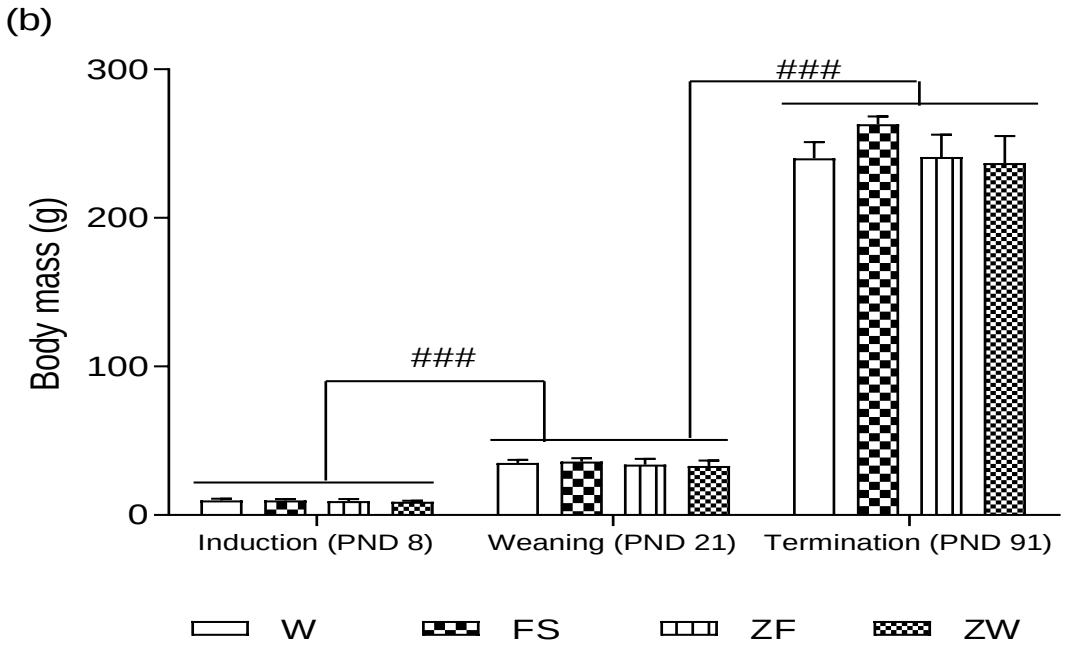
500
400
300
200
100
0
Body mass (g)
###
###
Induction (PND 8)
Weaning (PND 21)
Termination (PND 91)
W
FS
ZF
ZW


(b)
300
200
100
0
Body mass (g)
###
###
Induction (PND 8)
Weaning (PND 21)
Termination (PND 91)
W
FS
ZF
ZW

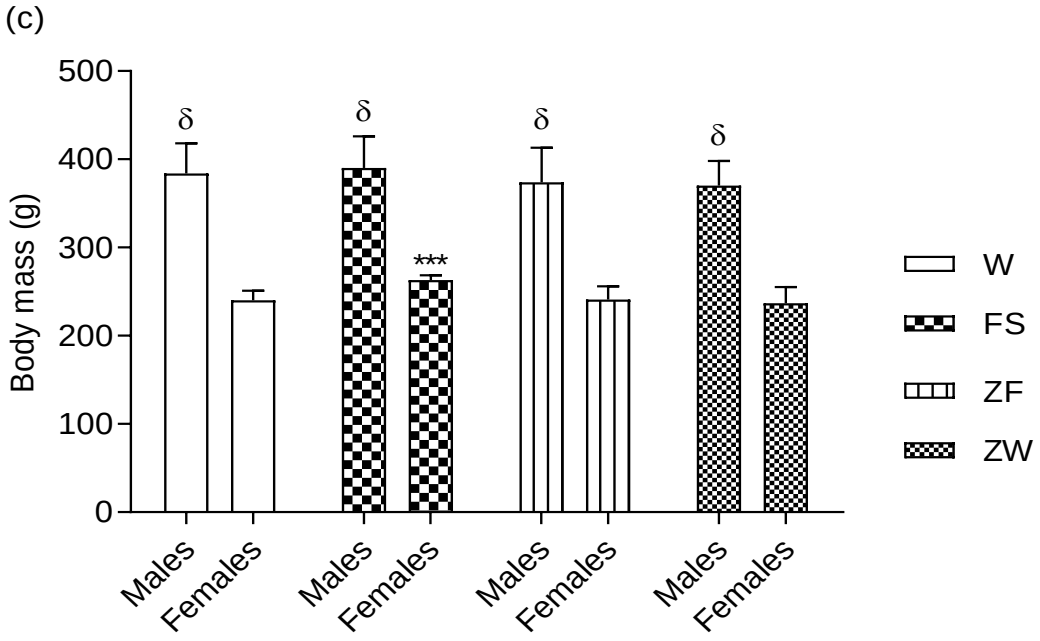


Figure 3.1: Effects of neonatally administered zingerone on average body mass of high-fructose fed male (a), and female (b) rats from induction to weaning and termination, and compared terminal mass between males and females (c).

*Data expressed as mean ± standard deviation. ### = P < 0.0001 when induction is compared to weaning and when weaning is compared to termination; *** = P < 0.0001 when FS termination mass is compared to other treatments in females; δ = P < 0.0001 when males' termination masses are compared to those of females; W = gavaged with 10 ml/kg body weight (bwt) of distilled water before weaning and then provided unlimited access to plain tap water post-weaning [Males = 9, Females = 11]; FS = gavaged with 10 ml/kg bwt of a 20% fructose solution before weaning and then provided unlimited access to a 20% fructose solution to drink post-weaning (Males = 9, Females = 11); ZF = gavaged with a combination of 20% fructose solution (10 ml/kg bwt) and zingerone (40 mg/kg bwt) before weaning and then unlimited access to 20% fructose solution to drink post-weaning (Males = 9, Females = 12); ZW = gavaged with zingerone only at 40 mg/kg bwt dissolved in distilled water before weaning and given unlimited access to plain tap water to drink post-weaning (Males = 8, Females = 10); PND = postnatal day.*

3.4.3 Clinical metabolic parameters in circulation

Table 3.1 shows the plasma lipid profile of the study rats. In both males and females, the high-fructose diet (FS) significantly increased plasma triglyceride concentrations [$P = 0.0226$ (males); $P = 0.0049$ (females)], triglycerides to HDL-c ratio [$P = 0.0190$ (males); $P = 0.0084$ (females)], concentrations of TC [$P = 0.0970$ (males); $P = 0.0005$ (females)] and LDL-c [$P = 0.0002$ (males); $P < 0.0001$ (females)] compared to the control (W; Table 3.1). The high-fructose diet-induced hypercholesterolemia (both sexes), hyper-LDL-cholesterolaemia (both sexes) and increase in triglycerides to HDL-c ratio (females only) were prevented ($P > 0.05$ when ZF was compared to control) by the neonatal orally administered zingerone (Table 3.1; with zingerone treated groups having similar concentrations of TC, LDL-c and triglycerides to HDL-c ratio with the control). The concentrations of HDL-c [Table 3.1; $P = 0.2847$ (males); $P = 0.3765$ (females)], fasting glucose [$P = 0.1042$ (males); $P = 0.3679$ (females)], insulin [$P = 0.3387$ (males); $P = 0.6824$ (females)] and adiponectin [$P = 0.4289$ (males); $P = 0.9563$ (females)], as well as the values of HOMA-IR [$P = 0.3033$ (males); $P = 0.9208$ (females)] were not different across the different treatment groups (Table 3.2) in both sexes.

Females rats had significantly higher levels of total [$P < 0.0001$ (sex and treatment effects), $P = 0.9590$ (interaction effect)], LDL [$P < 0.0001$ (sex and treatment effects), $P = 0.3259$ (interaction effect)] and HDL [$P = 0.0124$ (sex effect), $P = 0.0004$ (treatment and interaction effects)] cholesterols than their corresponding males. However, no sex difference was observed in the levels of TGs [$P = 0.2117$ (sex effect), $P < 0.0001$ (treatment effect), $P = 0.4545$ (interaction effect)] TGs to HDL-c ratio [$P = 0.1628$ (sex effect), $P = 0.0002$ (treatment effect), $P = 0.4530$ (interaction effect)] and glycaemic parameters ($P > 0.05$ for sex, treatment and interaction effects).

Table 3.1: Effects of neonatally administered zingerone on lipid profile of high-fructose fed rats in adulthood

Parameter	Sex	W	FS	ZF	ZW
TGs (mmol/l)	Male	0.55 ± 0.27^{a}	1.30 ± 0.52^{b}	0.82 ± 0.38^{ab}	0.79 ± 0.65^{ab}
	Female	0.41 ± 0.27^{a}	1.10 ± 0.69^{b}	0.96 ± 0.57^{ab}	0.43 ± 0.35^{a}
TC (mmol/l)	Male	5.40 ± 1.60^{a}	8.80 ± 2.40^{b}	5.60 ± 1.80^{a}	6.10 ± 2.90^{ab}
	Female	$8.10 \pm 2.40^{a\lambda}$	$11.00 \pm 2.50^{b\lambda}$	$7.60 \pm 1.70^{a\lambda}$	$8.20 \pm 1.90^{ab\lambda}$
HDL-c (mmol/l)	Male	1.70 ± 0.06^{a}	1.70 ± 0.04^{a}	1.70 ± 0.05^{a}	1.70 ± 0.04^{a}
	Female	$1.70 \pm 0.04^{a\lambda}$	$1.70 \pm 0.05^{a\lambda}$	$1.80 \pm 0.04^{a\lambda}$	$1.70 \pm 0.03^{a\lambda}$
LDL-c (mmol/l)	Male	3.70 ± 0.75^{a}	5.00 ± 0.26^{b}	3.60 ± 0.71^{a}	4.00 ± 0.59^{a}
	Female	$4.80 \pm 0.48^{a\lambda}$	$6.50 \pm 1.30^{b\lambda}$	$4.30 \pm 0.43^{a\lambda}$	$4.90 \pm 0.52^{a\lambda}$
TGs : HDL-c ratio	Male	0.32 ± 0.16^{a}	0.75 ± 0.30^{b}	0.48 ± 0.23^{ab}	0.47 ± 0.38^{ab}
	Female	0.24 ± 0.16^{a}	0.61 ± 0.40^{b}	0.55 ± 0.33^{ab}	0.25 ± 0.21^{a}

Data expressed as mean ± standard deviation. ^{ab} = within row means with different letters significantly different at $P < 0.05$; $^{\lambda}$ = female rats had significantly higher level of cholesterol subtypes at $P < 0.0001$ compared to males; W = gavaged with 10 ml/kg body weight (bwt) of distilled water before weaning and then provided unlimited access to plain tap water post-weaning [Males (M) = 9, Females (F) = 11]; FS = gavaged with 10 ml/kg bwt of a 20% fructose solution before weaning and then provided unlimited access to a 20% fructose solution to drink post-weaning (M = 9, F = 11); ZF = gavaged with a combination of 20% fructose solution (10 ml/kg bwt) and zingerone (40 mg/kg bwt) before weaning and then unlimited access to 20% fructose solution to drink post-weaning (M = 9, F =12); ZW = gavaged with zingerone only at 40 mg/kg bwt dissolved in distilled water before weaning and given unlimited access to plain tap water to drink post-weaning (M = 8, F = 10); TGs = triglycerides; TC = total cholesterol; HDL-c = high density lipoprotein cholesterol; LDL-c = low density lipoprotein cholesterol.

Table 3.2: Effects of neonatally administered zingerone on glycaemic parameters of high-fructose fed rats in adulthood

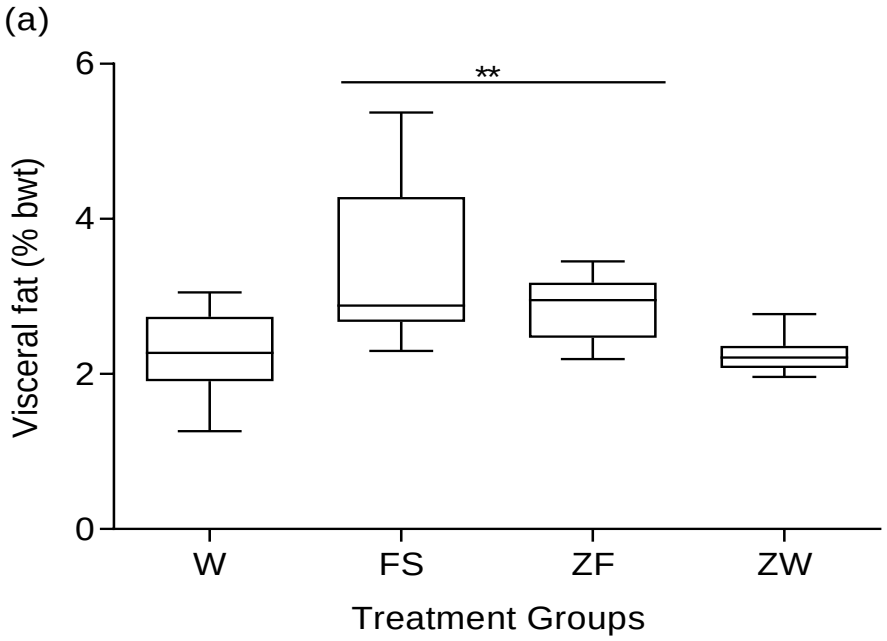
Parameter	Sex	W	FS	ZF	ZW
Glucose	Male	4.10 ± 0.38	3.80 ± 0.47	3.70 ± 0.18	4.10 ± 0.25
(mmol/l)	Female	3.80 ± 0.47	3.70 ± 0.47	3.60 ± 0.42	3.90 ± 0.34
Insulin	Male	0.85 ± 0.70	1.40 ± 0.58	1.20 ± 0.48	1.20 ± 0.63
(ng/ml)	Female	1.00 ± 0.61	1.10 ± 0.46	1.20 ± 0.43	0.93 ± 0.62
HOMA-IR	Male	0.15 ± 0.12	0.24 ± 0.12	0.20 ± 0.08	0.22 ± 0.11
index	Female	0.17 ± 0.12	0.18 ± 0.09	0.19 ± 0.06	0.16 ± 0.11
Adiponectin	Male	176.00 ± 99.00	275.00 ± 107.00	293.00 ± 221.00	253.00 ± 179.00
(pg/ml)	Female	243.00 ± 106.00	228.00 ± 151.00	256.00 ± 140.00	232.00 ± 119.00

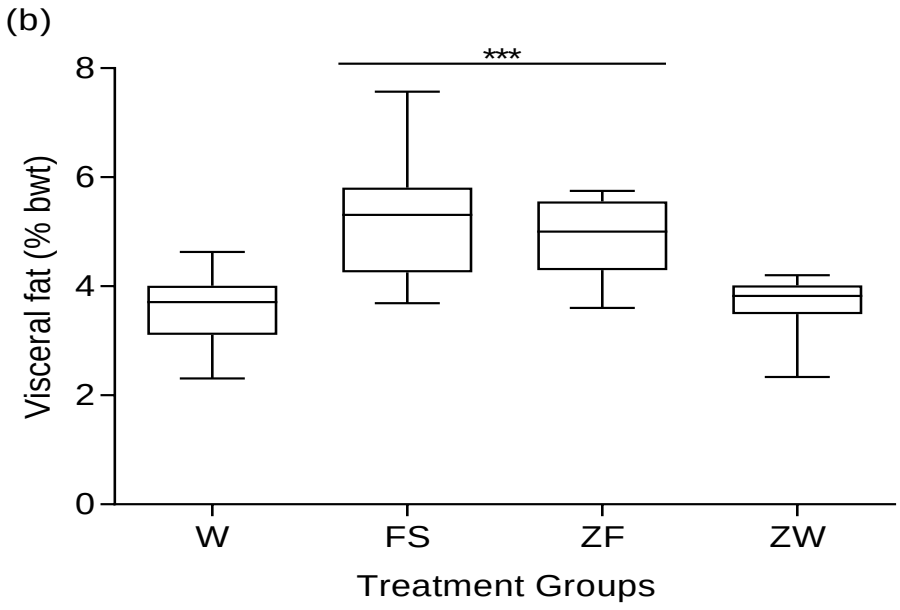
Data expressed as mean ± standard deviation. P > 0.05; W = gavaged with 10 ml/kg body weight (bwt) of distilled water before weaning and then provided unlimited access to plain tap water post-weaning [Males (M) = 9, Females (F) = 11]; FS = gavaged with 10 ml/kg bwt of a 20% fructose solution before weaning and then provided unlimited access to a 20% fructose solution to drink post-weaning (M = 9, F = 11); ZF = gavaged with 10 ml/kg bwt of a 20% fructose solution combined with zingerone at 40 mg/kg bwt before weaning and then unlimited access to 20% fructose solution to drink post-weaning (M = 9, F = 12); ZW = gavaged with zingerone only at 40 mg/kg bwt dissolved in distilled water before weaning and given unlimited access to plain tap water to drink post-weaning (M = 8, F = 10); HOMA-IR = homeostatic model assessment of insulin resistance.

3.4.4 Visceral obesity

The visceral fat percentages of both male and female rats are shown in Figure 3.2. In both sexes, high dietary fructose (FS; ZF) significantly [$P = 0.0037$ (males); $P < 0.0001$ (females)] increased visceral fat (percentage body mass) compared to the control (W; Figure 3.2a and 3.2b). However, the zingerone only treated group (ZW) had similar relative visceral fat percentage with the controls in both sexes [$P = 0.1590$ (males); $P = 0.3414$ (females)].

Female rats significantly [$P < 0.0001$ (sex and treatment effects), $P = 0.3573$ (interaction effect) had higher visceral fat percentage than males (Figure 3.2c).


(a)
**
Visceral fat (% bwt)
6
4
2
0
W
FS
ZF
ZW
Treatment Groups


(b)
***
Visceral fat (% bwt)
8
6
4
2
0
W
FS
ZF
ZW
Treatment Groups

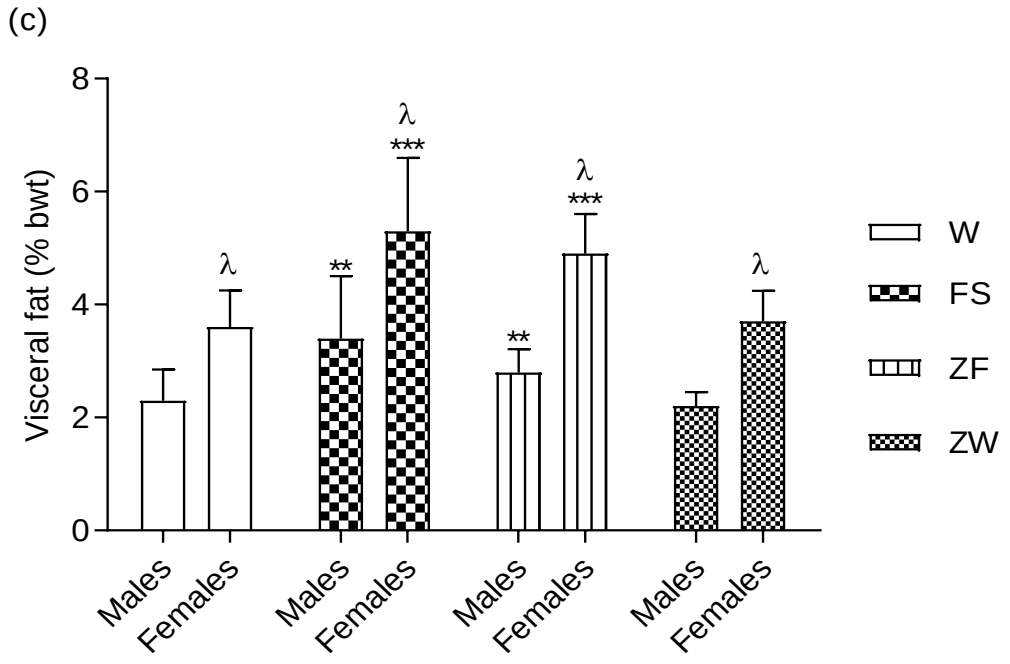


Figure 3.2: Effects of neonatally administered zingerone on the relative mass of visceral fat percentage (% bwt) of high-fructose fed male (a) and female (b) rats in adulthood; and visceral fat comparism between males and females (c).

*Data expressed as mean ± standard deviation. ** = fructose treated groups in male rats (FS; ZF) had significantly higher visceral fat percentage at P = 0.0037 when compared to other treatments; *** = fructose treated groups in female rats (FS; ZF) had significantly higher visceral fat percentage at P < 0.0001 when compared to other treatments; λ = female rats had significantly higher visceral fat percentage at P < 0.0001 compared to males; W = gavaged with 10 ml/kg body weight (bwt) of distilled water before weaning and then provided unlimited access to plain tap water post-weaning [Males (M) = 9, Females (F) = 11]; FS = gavaged with 10 ml/kg bwt of a 20% fructose solution before weaning and then provided unlimited access to a 20% fructose solution to drink post-weaning (M = 9, F = 11); ZF = gavaged with a combination of 20% fructose solution (10 ml/kg bwt) and zingerone (40 mg/kg bwt) before weaning and then unlimited access 20% fructose solution to drink post-weaning (M = 9, F = 12); ZW = gavaged with zingerone only at 40 mg/kg bwt dissolved in distilled water before weaning and given unlimited access to plain tap water to drink post-weaning (M = 8, F = 10); % bwt = percentage body weight.*

3.5 Discussion

The study evaluated whether orally administered zingerone in the neonatal period would protect against the development later in adulthood, of long-term dietary fructose-induced metabolic derangements in Sprague Dawley rats. In both sexes, it was observed that as adults, there were differences in fluid consumption such that rats provided fructose solution drank more fluid and had a greater total calorie intake than those that had plain water. Additionally, the rats provided fructose solution as drinking fluid ate less food (standard rat chow) than those provided plain water. Rats provided fructose solution to drink had increased terminal body masses (females, and not males), visceral adiposity and elevated plasma triglyceride and cholesterol concentrations at termination (both sexes). Administration of zingerone to neonatal rats programmed protection against high-fructose diet-induced increase in body mass (females, and not males) and hypercholesterolemia in adulthood (both sexes). Dimorphic responses were also observed with the manifestation of metabolic parameters.

The amount of food or water that is consumed by an organism depends on several intrinsic and extrinsic factors, including palatability, the aroma of the food and state of satiety [13]. The current study reports that the rats that received fructose solution consumed larger amounts of fluid (FS), but lesser amounts of rat chow when compared with those that did not receive fructose solution as drinking fluid in adult life in both sexes (Supplementary Tables S1A and S1B). The increase in fluid intake observed in the high-fructose diet-fed rats could have been due to the sweet taste of the fructose solution [10,29]. However, rats have intrinsic mechanisms that regulate calorie intake [13]. This could explain why there was a reduction in the consumption of solid food by the rats drinking fructose in order to maintain their calorie intake, preferentially from consumption of the fructose solution. In addition, 5 mls of 20% fructose solution (which contains 1 g fructose) yields more calories of energy than the 1 g of standard rat food [29]. Therefore, despite the food consumption being higher in the rats that received water (W and ZW), the rats that received fructose had a relatively higher caloric intake from fructose than the other rats (Supplementary Table S1C).

It was observed in this study that female rats consumed relatively more food and fluid (and thus had more calories) than males in adulthood. Dimorphic responses have been reported on rats' feeding, with females having higher intake than males [33]. The increase in food intake observed in

females could be due to increase in ghrelin levels that is reported to be higher in females [33]. However, we did not assay ghrelin levels in this study. In addition, females have a been reported to have a greater preference for sweet solutions than males [34]. This could probably be due to oestrogen effects on the satiety centre [35]. An increase in fructose consumption could lead to metabolic complications including increasing the body mass of the rats.

The induction body mass of the rats was not different across the treatments in both sexes. This reflects the deliberate choice to use rats born in litter sizes of 8-12, thereby avoiding the impact of litter size on induction mass which could impact on the study outcomes, as small litter sizes are prone to faster growth and obesity compared to large litter sizes [36]. The suckling period is characterised by rapid growth which can be altered (positively or negatively) by dietary interventions [37]. The high-fructose diet and/or zingerone had no effect on the weaning (PND 21) body mass of the male and female pups in this study, suggesting that both fructose and zingerone did not negatively affect growth during the neonatal period. Although the terminal body mass of the male rats was not affected by the interventions, the high-fructose diet increased the terminal body mass of the female rats compared to the other rats. Zingerone programmed protection against the high-fructose diet-induced increase in body mass in female rats. Fructose is known to increase body mass by increasing hepatic *de novo* lipogenesis and tissue's adipogenesis that contribute to the development of obesity [11]. Thus, it could be speculated that neonatal orally administered zingerone prevented the fructose-induced increase in terminal body masses by programming protection (via regulating hepatic fatty acid oxidation) against fructose-induced hepatic lipogenesis and tissue's adipogenesis in the female rats [38]. The higher terminal body mass observed in males in comparison to females could be due to the greater trophic effects of testosterone and growth hormone in males compared to females [39,40]. An increase in body mass is associated with visceral obesity that is known to result in poor cardio-metabolic health [41].

The present study found that a long-term high-fructose diet had programmed for increased visceral adiposity in both male and female rats. The high-fructose diet-fed rats that were administered neonatal zingerone had a 26% (males) and 11% (females) reduction in mean visceral fat pad mass compared to those who were fed the high-fructose diet alone. This means that neonatal zingerone had programmed for a reduction in the visceral adiposity observed with fructose fed rats. It has been reported that complete attenuation of obesity is not usually

achievable in practical situations, however, a 5-10% weight loss significantly decreases the risk of morbidity and mortality [42]. Although not significantly different statistically, an 11% reduction in the visceral fat showed that neonatal zingerone could potentially attenuate the risk of developing cardio-metabolic complications later in life, since increased visceral obesity has been linked to the development of cardio-metabolic disorders including dyslipidaemia [41].

The higher visceral fat percentage observed in female rats than males could be explained by the fact that females have a greater tendency of fat accumulation than males, an effect that has been ascribed to female sex hormones [43]. This finding had reinforced that of terminal body masses observed in both sexes, in which fructose had increased the terminal body mass in females but not in males. An increase in adiposity is invariably associated with dyslipidaemia [41].

Indeed, in the current study, the long-term intake of a high-fructose diet resulted in hypertriglyceridaemia, hypercholesterolemia, hyper-LDL-cholesterolemia in both sexes. Dyslipidaemia is a key component of metabolic syndrome that can lead to cardiovascular diseases including hypertension and atherosclerosis [12]. The triglyceride to HDL-cholesterol ratio is an atherogenic index that is used to assess the risk of developing metabolic syndrome and coronary artery disease [44]. This study found that long-term intake of a high-fructose diet increased the triglycerides to HDL-cholesterol ratio in adulthood suggesting a greater risk of developing metabolic syndrome and related cardiovascular diseases [45]. Neonatal zingerone prevented the fructose-induced dyslipidaemia, thus zingerone had programmed protection against fructose-induced dyslipidaemia in adulthood. These anti-hyperlipidaemic effects of zingerone have been observed in streptozotocin-induced diabetic adult rats [23]. It was reported that zingerone exerts its anti-hyperlipidaemic effects by stimulating lipolysis and enhancing the activity of norepinephrine-sensitive lipases [46]. We observed sex differences in the levels of cholesterol subtypes such that cholesterol was elevated in female rats in this study. This is not surprising since females are more prone to dyslipidaemia than males, which could have been due to oestrogen effect [43]. Fructose-induced dyslipidaemia can result without affecting glycaemic parameters in rats [47].

Despite fructose consumption having been implicated in the aetiology of hyperglycaemia, hyperinsulinaemia, insulin resistance and hypoadiponectinaemia in adult rats [48], these parameters were not affected by the interventions of the current study, in both sexes. This indicated that

administration of 20% fructose solution did not programme for the development of hyperglycaemia, hyperinsulinaemia, insulin resistance and low plasma adiponectin in adulthood. These findings contradict that of Ibitoye and Ajiboye [48] who reported dysglycaemia and hypoadiponectinaemia following administration of a high fructose diet to adult Wistar rats for 6 weeks. The variance could be due to the rats' age at commencement of the study, strain and the mode of fructose administration. High-fructose diets where fructose is mixed into feed tend to produce features of metabolic dysfuction more readily than fructose intake as a drinking solution [49]; likewise, Wistar rats are more prone to manifest features of metabolic syndrome than Sprague Dawley rats [29]. Older rats are also more susceptible to the effects of fructose than younger rats due to innate protective mechanisms present at young age [49]. As consumption of fructose did not affect the glycaemic parameters, the potential protective programming effect of neonatal zingerone against dysglycaemia and insulin resistance in adulthood could not apparently manifest, and this needs future investigation.

Our study is novel in showing that zingerone administered in the neonatal phase can programme long-term protection against high-fructose diet-induced dyslipidaemia. The precise mechanisms need to be further explored however, it is likely that zingerone could have dysregulated the proteins and receptors involved in lipid metabolism such as PPARα thereby regulating fatty acid oxidation and preventing the fructose-induced lipogenesis and dyslipidaemia [25]. As mentioned in the introduction section, the liver also plays a central role in lipid metabolism, and the impact of the interventions on the liver needs to be investigated in future studies.

3.6 Conclusion

This study has demonstrated that long-term fructose intake caused visceral obesity and dyslipidaemia in both male and female rats. The novelty of our study is that oral administration of zingerone to rats during the neonatal period programmed for long-term protection against the development of high-fructose diet-induced visceral obesity and dyslipidaemia later in adult life. Thus, a similar strategic use of zingerone neonatally could be explored in humans to manage diet-induced metabolic syndrome, thereby replacing the use of animal models in exploring the therapeutic effects of zingerone.

3.7 References

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The next chapter focuses on the potential protective effects of neonatally administered zingerone against the long-term development of fructose-induced non-alcoholic fatty liver disease, a component considered as hepatic manifestation of metabolic syndrome.

CHAPTER FOUR - Published manuscript 2

Zingerone administered neonatally prevents the subsequent development of high dietary fructose-induced fatty liver in Sprague Dawley rats

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(2021)

Journal of Medicinal Food, pp 1-9. doi: 10.1089/jmf.2020.0189

4.1 Abstract

Consumption of high-fructose diets early in life increases the risk of developing metabolic disorders, including non-alcoholic fatty liver disease (NAFLD). Zingerone, an alkaloid isolated from *Zingiber officinale* has been demonstrated to reverse obesity and fatty liver in adult male rats. We investigated the potential preventive effects of neonatally administered zingerone on the development of fructose-induced NAFLD in male and female rats.

Four-day old male (n = 35) and female (n = 44) rat pups were randomized and gavaged with: 10 ml/kg body weight (bwt) of distilled water (C), 10 ml/kg bwt of 20% fructose solution (Fr), 10 ml/kg bwt of 20% fructose solution + 40 mg/kg bwt of zingerone (ZFr), and 40 mg/kg bwt of zingerone (Z) daily for 14 days. After weaning, all groups continued on unlimited standard rat feed, however groups C and Z had plain drinking water while groups Fr and ZFr had unlimited 20% fructose solution to drink for 10 weeks.

Rats on the high-fructose diet (Fr) compared to the negative controls (C) had significantly increased hepatic lipid content [(%), $P = 0.0002$ (Males); $P < 0.0001$ (Females), ANOVA] and hepatic steatosis score [(%), $P = 0.0018$ (Males); $P < 0.0022$ (Females), Kruskal-Wallis ANOVA]. Zingerone prevented ($P < 0.05$) the fructose-induced increase in hepatic steatosis in both sexes. The plasma ALT activity, levels of uric acid, TBARS, IL-6 and TNF-α were not different ($P > 0.05$, ANOVA) across the different treatment groups in both sexes. No difference ($P > 0.05$, ANOVA) was observed between the two sexes for treatment, sex and interaction effects with regard to hepatic lipid content, and measured blood parameters.

The use of zingerone neonatally should be further investigated as a strategic prophylactic intervention for the prevention of long-term high-fructose diet-induced NAFLD.

Keywords: Early postnatal period, NAFLD, Sprague Dawleys, sugary diets, vanillyl acetone

4.2 Introduction

The average global prevalence of non-alcoholic fatty liver disease (NAFLD) is 25% [1]. This prevalence is higher in males compared to females [2]. NAFLD is a clinical condition characterized by fatty infiltration of hepatocytes in the absence of significant alcohol consumption [3]. Histologically, NAFLD initially presents as simple steatosis which in 10-15% of patients, progresses to non-alcoholic steatohepatitis (NASH) [3]. NASH is characterized by inflammation,

and can further progress to fibrosis and eventually cirrhosis of the liver leading to hepatocellular carcinoma [4]. NAFLD is asymptomatic in most patients but associated with obesity and risk factors of metabolic syndrome [5]. It is the commonest cause of chronic liver disease globally [5]. Despite limited data on the global economic burden of managing NAFLD, the annual medical cost of managing NAFLD is approximately 62 billion dollars in the United States [6], and the cost of liver transplants is expected to rise with increasing prevalence of the condition [7].

Despite NAFLD being associated with the components of metabolic syndrome (obesity, dyslipidemia or insulin resistance) [8], recent evidence shows that NAFLD can develop independently, in the absence of the aforementioned metabolic disorders [9]. Nutrition, environment, gut microbiota and genetics/epigenetics are amongst the factors implicated in the pathogenesis of NAFLD [4].

Environmental and nutritional influences during periods of developmental plasticity such as the neonatal period, can result in epigenetic changes which can alter phenotype physiology and susceptibility to the development of metabolic diseases, including NAFLD, later in life [10]. Excessive intake of fructose during the perinatal period may serve to sensitize the liver to subsequent nutritional, metabolic and environmental events which trigger the development of NAFLD, hepatomegaly and increase in general body mass later in life [4,11,12]. Additionally, high dietary fructose consumption is a major cause of NAFLD due to the unregulated nature of fructose metabolism [13].

Prolonged excessive hepatic fructose metabolism causes adenosine triphosphate (ATP) depletion which stimulates uric acid synthesis leading to mitochondrial dysfunction and oxidative stress [13]. These events stimulate the production of reactive oxygen species (ROS) that eventually lead to inflammation characterized by elevated pro-inflammatory cytokines, apoptosis and fibrosis [4]. Metabolism of fructose also affects the expression of genes and receptors involved in the different steps of hepatic lipid accumulation and hepatic inflammation [14]. These genes and receptors include those associated with carbohydrate response element-binding protein (ChREBP), sterol regulatory element binding protein 1 (SREBP-1), acetyl-CoA carboxylase 1 (ACC-1), carnitine palmitoyltransferase 1 (CPT1), tumour necrosis factor alpha (TNFα) and peroxisome proliferator-activated receptor alpha (PPARα) [14]. Dysregulation of these genes and

receptors can lead to the development of NAFLD secondary to hepatic lipid accumulation and inflammation [4].

The inflammatory, apoptotic and lipid peroxidation processes associated with NAFLD can generate TNF-α, interleukin-6 (IL-6) and thiobarbituric reactive substances (TBARS) [3] which damage hepatocytes leading to elevation of key liver enzymes in circulation, particularly alanine aminotransferase (ALT); though this elevation does not correlate with the extent of steatosis, inflammation or fibrosis [5]. NAFLD can be managed effectively in its early stages of pathological development.

Despite no specifically approved pharmacological drug treatment for NAFLD [1], regular exercise and good dietary habits are the first line approaches in the early management of NAFLD [15]. The use of synthetic pharmacological agents like fenofibrate used in the management of specific risk factors of metabolic syndrome, is limited as these agents are usually expensive, require chronic use, inaccessible in some communities and monotherapeutic [16,17]. There is need for innovative prophylactic interventions against NAFLD using locally available resources.

Globally, communities are increasingly opting for plant-derived medications for their healthcare needs [16]. The medicinal potential of natural agents for protection against adverse neonatal metabolic programming of NAFLD needs to be explored. Previous studies have used fructose-fed rats during the immediate postnatal period to programme for long-term metabolic health outcomes using phytochemicals. Neonatal administration of Ursolic acid [18], Oleanolic acid [19], and S-allyl cysteine [20] prevented the development of hepatic lipid accumulation, in rats fed high-fructose diets. In the current study, we investigated whether zingerone could be used for that purpose.

Zingerone (vanillyl acetone) is an alkaloid found in the rhizomes of the plant ginger (*Zingiber officinale*). Studies have documented the hepatoprotective and antiobesity properties of zingerone in adult rat and mouse experimental models of metabolic disorders [21–24]. The mechanisms by which zingerone exerts its hepatoprotective effects include its ability to upregulate lipolysis, scavenge free radicals and downregulate inflammatory cytokines in the liver [21,24].

Given the heavy burden that management of NAFLD places on healthcare systems globally and the absence of specific treatments for NAFLD, there is need for strategic prophylactic

interventions. The biological properties of zingerone made it a suitable candidate phytochemical in the current study for administration during the neonatal period to explore its potential to protect against long-term development of NAFLD secondary to high-fructose diets.

4.3 Materials and Methods

4.3.1 Experimental setting and procedures

The study was approved by the Animal Research Ethics Committee (AREC) of the University of Witwatersrand (Clearance No: 2017/010/71/B), and conducted at the Central Animal Service (CAS) of the University of Witwatersrand, Johannesburg, South Africa, using internationally accepted principles for laboratory animal use and care [South African National Standard (SANS 10386:2008) and Animals Protection Act, 1962: Act No. 71]. The Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines were adopted while compiling the manuscript.

The rats used in this study were obtained from the CAS, University of the Witwatersrand. Seven nulliparous dams of Sprague-Dawley rats (*Rattus norvegicus*), each with a litter consisting of 8-12 four-day-old suckling male and female pups weighing 9.7 ± 1.1 g were used in this study. The dams with their respective litters were housed in Perspex cages containing wood shavings as bedding. The room temperature was maintained at 25 ± 1 °C and a 12-h light and dark cycle with lights off from 7pm to 7am. The dams were supplied with standard rat chow (LabChef Rodent Breeder, (Pty) Ltd, Stellenbosch, South Africa) and *ad libitum* water access throughout the suckling period. The pups were allowed to freely nurse from their respective dams until weaning on postnatal day 21. The dams were returned to stock after weaning of their pups. The weaned rats were then housed individually in Perspex cages having the same environmental conditions described earlier.

The study consisted of two main stages (Figure 4.1): A pre-weaning stage (postnatal day 8-21) preceded by a four-day adaptation period, and a post-weaning stage (postnatal day 22-91). On postnatal day 8, the pups (n = 79; 35 males, 44 females) from each litter were randomly allocated to four treatment groups: **Group 1**: Received 10 ml/kg body weight (bwt) of distilled water gavaged orally before weaning (daily) and *ad libitum* access to plain drinking tap water after weaning (9 males, 11 females); **Group 2:** Received 10 ml/kg bwt of a 20% fructose solution gavaged orally before weaning (daily) and *ad libitum* access to a 20% drinking fructose solution after weaning (9 males, 11 females) to induce metabolic dysfunction; **Group 3:** In which we investigated whether neonatal

zingerone could programme for protection against long-term high fructose consumption, received 10 ml/kg bwt of a 20% fructose solution + zingerone at 40 mg/kg bwt gavaged orally before weaning (daily) and *ad libitum* access to a 20% drinking fructose solution after weaning (9 males, 12 females); **Group 4:** Received 40 mg/kg bwt of zingerone dissolved in 10 ml/kg bwt of distilled water gavaged orally before weaning (daily) and *ad libitum* access to plain drinking tap water after weaning (8 males, 10 females) to assess the effects of zingerone alone on metabolic health of the animals.

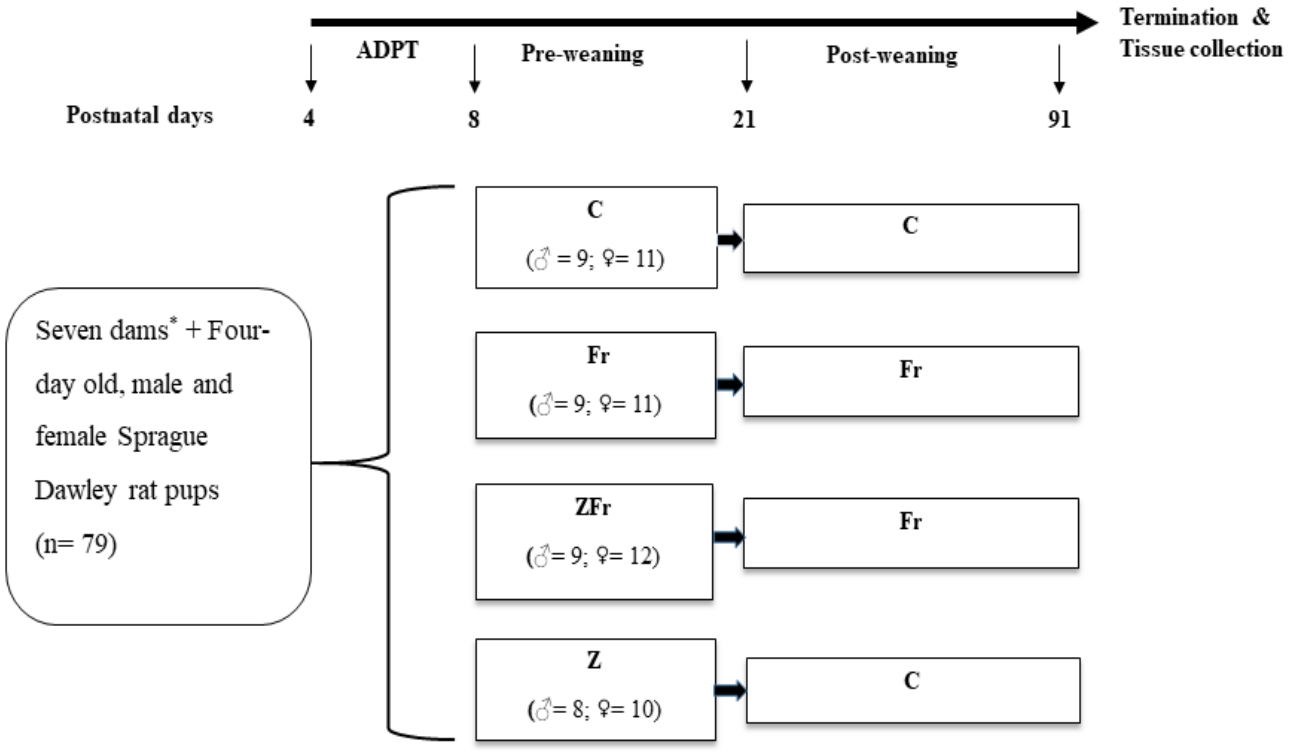

Figure 4.1: Flow diagram showing the timeline and treatment groups.

** = only with pups during the adaptation and pre-weaning phases; C = plain water; Fr = 20% fructose solution; ZFr = zingerone (40 mg/kg bwt) + 20% fructose solution; Z = zingerone (40 mg/kg bwt); ADPT = adaptation; ♂ = males; ♀ = females.*

Fructose solution at 20% has been shown to induce metabolic dysfunction in rats [18]; whilst zingerone at 40 mg/kg bwt has been shown to prevent against ethanol-induced hepatotoxicity in rats [25], hence these doses were chosen for the current study.

The fructose solution (Natures Choice, South Africa) was constituted to 20% by adding 20 g of fructose into 100 ml of water [26], while food grade zingerone (catalogue number W312401-1KG) was purchased from Sigma-Aldrich (USA).

The rats were weighed daily during the pre-weaning stage and then twice a week during the post-weaning stage by individually placing the rats in a pre-weighed cage on an electronic balance (Snowrex Electronic Scale, Clover Scales, Johannesburg).

4.3.2 Procedures at termination

After the 12-week intervention period, the rats were fasted overnight, terminal body mass measured and then euthanised with an appropriate and acceptable euthanasia in rats, an intraperitoneal injection of sodium pentobarbitone [27] (Centaur Laboratories, Johannesburg, South Africa) at 150 mg/kg body weight. After euthanasia, a ventral midline incision was made through the thorax and abdomen; syringes with attached hypodermic needles were used to withdraw blood samples via intra-cardiac access. The blood samples were then transferred into heparinised tubes (BD Vacutainer, Plymouth, UK) and centrifuged (Rotofix 32A, Hettich Zentrifugen, Germany) at 3700 g for 15 minutes at 20 °C. The plasma was harvested and stored at -20 °C for determination of ALT, uric acid, and TBARS. The liver was carefully dissected out from the abdominal cavity and weighed using a Presica 310M balance (Precision Instruments, Johannesburg, South Africa)]. The right medial lobe of the liver was preserved in 10% phosphate buffered formalin for histological analysis, while the remaining liver lobes were preserved at -20 °C for quantification of lipid content.

4.3.3 Determination of hepato-somatic index

The terminal body mass and the fresh whole liver weight were used to compute the hepato-somatic index [28]:

Hepato-somatic index (%) = [mass of liver (g) / body mass (g)] × 100

4.3.4 Determination of hepatic lipid content

The hepatic lipids were determined by solvent extraction using a Soxhlet apparatus (Gebr. Rettberg GmbH, 37079 Göttingen, Germany), according to the Official Methods of Analysis of the Association of Official Agricultural Chemists (AOAC) International, 2005, Method 960.39. Briefly the liver samples were lyophilized in a freeze-dryer (VirTis BenchTop BK-FD12, SP

Scientific, NewYork, USA) and milled into a fine powder. The milled liver samples were then extracted using petroleum ether in a Soxhlet apparatus and the lipid quantified gravimetrically.

4.3.5 Histological assessment of the liver

The right medial lobes of the liver samples that were preserved in 10% phosphate buffered formalin were processed using an automatic tissue processor (Microm STP 120, ThermoScientific, MA, USA), embedded in paraffin wax, sectioned at 5µm and then stained with hematoxylin and eosin (H&E) for steatosis and inflammation assessment and Masson's trichrome (MT) for assessment of fibrosis. The slides were viewed under a light microscope (x40) (Leica Biosystems, USA) mounted to a camera which uses ZEISS ZEN microscope software for image capture, and scored for steatosis, collagen deposition and fibrosis.

The H&E images were used to score micro- and macro-vesicular steatoses as described by Kleiner et al.[29]. The grading was done based on the total area affected per camera field, according to the following criteria: Grade 0 steatosis: < 5% fat droplets; Grade 1 steatosis: 5–33% fat droplets; Grade 2 steatosis: 34–66% fat droplets and Grade 3 steatosis: > 66% per camera field at 40x magnification. Inflammation was scored by counting the foci of inflammatory cells in the liver parenchyma per camera field and graded as follows: Grade 0 = no foci of inflammation; Grade 1 = less than 2 foci; Grade 2 = 2–4 foci; Grade 3 = greater than 4 foci.

Fibrosis was assessed from photomicrographs of the MT-stained sections at ×40 magnification.

4.3.6 Determination of alanine aminotransferase, uric acid, thiobarbituric acid reactive substances and inflammatory markers in plasma

Plasma ALT activity and uric acid concentration were determined using a calibrated VetTest analyzer (IDEXX VetTest® Clinical Chemistry Analyzer, IDEXX Laboratories Inc., USA). TBARS, IL-6 and TNF-α were determined using rat-specific Enzyme Linked Immuno-Sorbent Assay kits (Elabscience Biotechnology Co., Ltd, Houston, Texas, USA) respectively.

4.3.7 Statistical analysis

Data were analysed using Graphpad Prism 8 software (Graph-pad Software Inc., San Diego, USA). Parametric data (hepato-somatic index, ALT, TBARS, IL-6, TNF-α and hepatic lipid content) were

analysed by one-way analysis of variance (ANOVA) and expressed as mean ± standard deviation (SD); followed by Tukey *post hoc* tests for comparison of the means. The Kruskal-Wallis test (non-parametric one-way ANOVA) was used to analyse multiple-group data for steatosis grades, inflammation and fibrosis; and expressed as median ± range; followed by Dunn's *post hoc* test to compare the medians. Sex variations were analysed using two-way ANOVA. Statistical significance was considered when $P < 0.05$.

4.4 Results

4.4.1 Hepato-somatic index and hepatic lipid accumulation

The mass of the liver relative to body weight was not different [Males (P = 0.6217); Females (P = 0.0657)] across the different treatment groups in both sexes (Table 4.1). No sex difference was observed between male and female rats regarding percentage liver weight [sex effect (P = 0.9999), treatment effect (P = 0.4540) and interaction effect (P = 0.3500).

Long-term fructose consumption significantly increased the hepatic lipid accumulation in both male (P = 0.0002) and female (P < 0.0001) rats compared to all other groups (Table 4.1). Thus, the neonatally administered zingerone prevented the fructose-induced hepatic lipid in both sexes, such that the zingerone treated (with or without fructose) group had a similar [P = 0.7632 (Males); P = 0.5103 (Females)] hepatic lipid content compared with negative controls. There was no sex difference observed [sex effect (P = 0.3774), treatment effect (P < 0.0001) and interaction effect (P = 0.8416) with regard to percentage hepatic lipids accumulation.

Table 4.1: Effects of neonatally administered zingerone on the hepato-somatic index and hepatic lipid accumulation of high-fructose diet fed rats

Parameter	Sex	C	Fr	ZFr	Z
Hepato-somatic Index (%)	Male	3.00 ± 0.21	3.00 ± 0.67	3.10 ± 0.15	3.00 ± 0.14
	Female	2.90 ± 0.14	3.10 ± 0.21	3.10 ± 0.20	3.00 ± 0.19
Hepatic lipids (% liver mass)	Male	4.50 ± 1.60^{a}	8.30 ± 2.20^{b}	5.20 ± 1.60^{a}	4.70 ± 1.70^{a}
	Female	4.50 ± 1.60^{a}	8.50 ± 2.40^{b}	6.20 ± 1.90^{a}	5.00 ± 1.60^{a}

Data expressed as mean ± standard deviation. ^{ab} *= row means with different letters (ab) significantly different at P = 0.0002 (Males) and P < 0.0001 (Females); C = 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 20; 9 males, 11 females); Fr = 10 ml/kg body weight of a 20% fructose solution before weaning and free access to a 20% fructose solution after weaning (n = 20; 9 males, 11 females); ZFr = 10 ml/kg body weight of a 20% fructose solution combined with zingerone at 40 mg/kg body weight before weaning and free access to a 20% fructose solution after weaning (n = 21; 9 males, 12 females); Z = zingerone only at 40 mg/kg body weight in 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 18; 8 males, 10 females).*

4.4.2 Histomorphometry of the liver

Figures 4.2A and B show representative photomicrographs of the liver histology from each of the experimental groups (H&E stain) of male and female rats respectively. There was a notable fatty infiltration (macro- and micro-vesicular steatosis indicated by solid and dash arrows respectively) in the sections of the fructose–only treated group which was generally absent in the other treatment groups [Figure 4.2A (Males); Figure 4.2B (Females)]. This was confirmed by the micro- and macro-vesicular steatosis scores which were greater in rats fed a high-fructose diet only compared to the other treatment groups in both sexes ($P < 0.05$, Dunn's multiple-comparison; Table 4.2). This demonstrated that zingerone prevented the development of steatosis induced by high-fructose diet in the study rats. No difference was observed between males and females on the steatosis score for sex ($P = 0.1889$), treatment ($P = 0.0826$) and interaction ($P = 0.7528$) effects. Additionally, there were no observable inflammatory cell aggregates in both sexes.

For the Masson's trichrome histological sections (MT stain), there was no observable fibrosis across the different treatment groups in both sexes [Figure 4.3A (Males); Figure 4.3B (Females)].

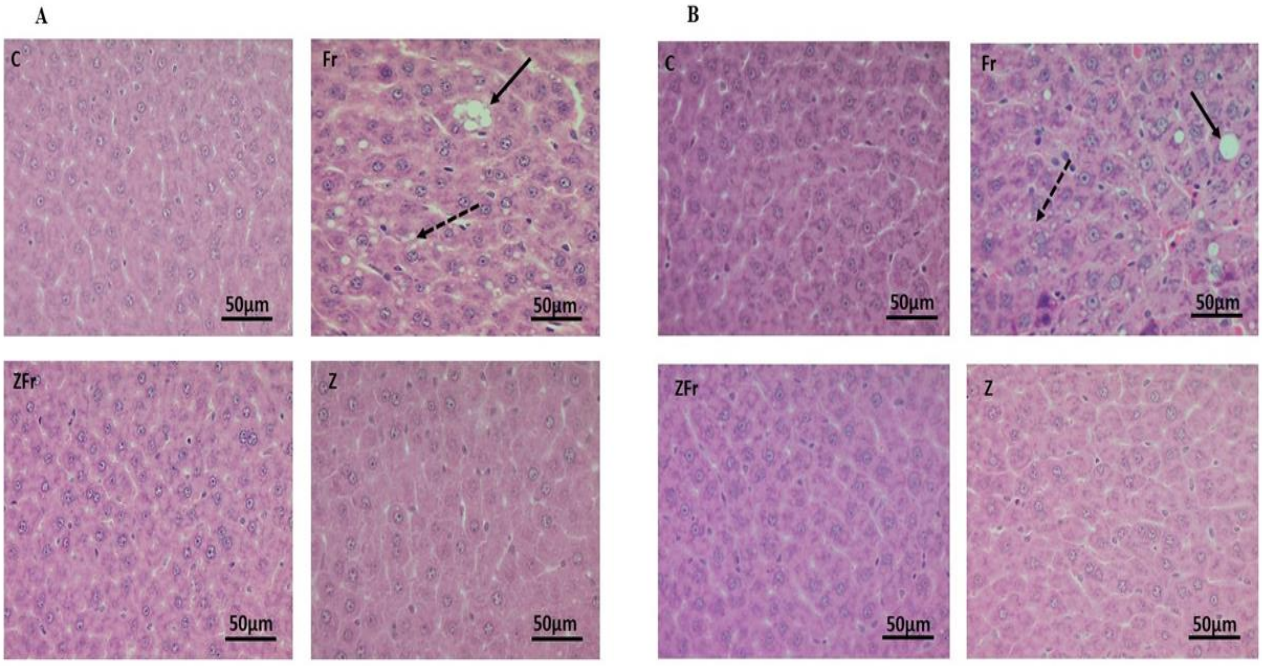

Figure 4.2: Representative photomicrographs of the male (Panel A) and female (panel B) rat liver sections stained with Hematoxylin and Eosin (x40).

C= 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n =20; 9 males, 11 females); Fr = 10 ml/kg body weight of a 20% fructose solution before weaning and free access to a 20% fructose solution after weaning (n = 20; 9 males, 11 females); ZFr = 10 ml/kg body weight of a 20% fructose solution combined with zingerone at 40 mg/kg body weight before weaning and free access to a 20% fructose solution after weaning (n =21; 9 males, 12 females); Z = zingerone only at 40 mg/kg body weight in 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 18; 8 males, 10 females); the dash arrows point to micro-steatosis; solid arrows point to macro-steatosis; scale bar = 50 µm.

Table 4.2: Effects of neonatally administered zingerone on the steatosis scores of high-fructose fed rats

Parameter	Sex	C	Fr	ZFr	Z
Micro-steatosis	Male	0 (0, 0)^{a}	1 (0, 3)^{b}	0 (0, 0)^{a}	0 (0, 0)^{a}
	Female	0 (0, 2)^{a}	2 (0, 3)^{b}	0 (0, 1)^{a}	0 (0, 2)^{a}
Macro-steatosis	Male	0 (0, 1)^{a}	1 (0, 2)^{b}	0 (0, 1)^{a}	0 (0, 1)^{a}
	Female	0 (0, 0)^{a}	1 (0, 2)^{b}	0 (0, 0)^{a}	0 (0, 1)^{a}

Data expressed as median and interquartile range ^{ab} = row means with different letters (ab) significantly different at P < 0.05; C = 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 20; 9 males, 11 females); Fr = 10 ml/kg body weight of a 20% fructose solution before weaning and free access to a 20% fructose solution after weaning (n = 20; 9 males, 11 females); ZFr = 10 ml/kg body weight of a 20% fructose solution combined with zingerone at 40 mg/kg body weight before weaning and free access to a 20% fructose solution after weaning (n =21; 9 males, 12 females); Z = zingerone only at 40 mg/kg body weight in 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 18; 8 males, 10 females).

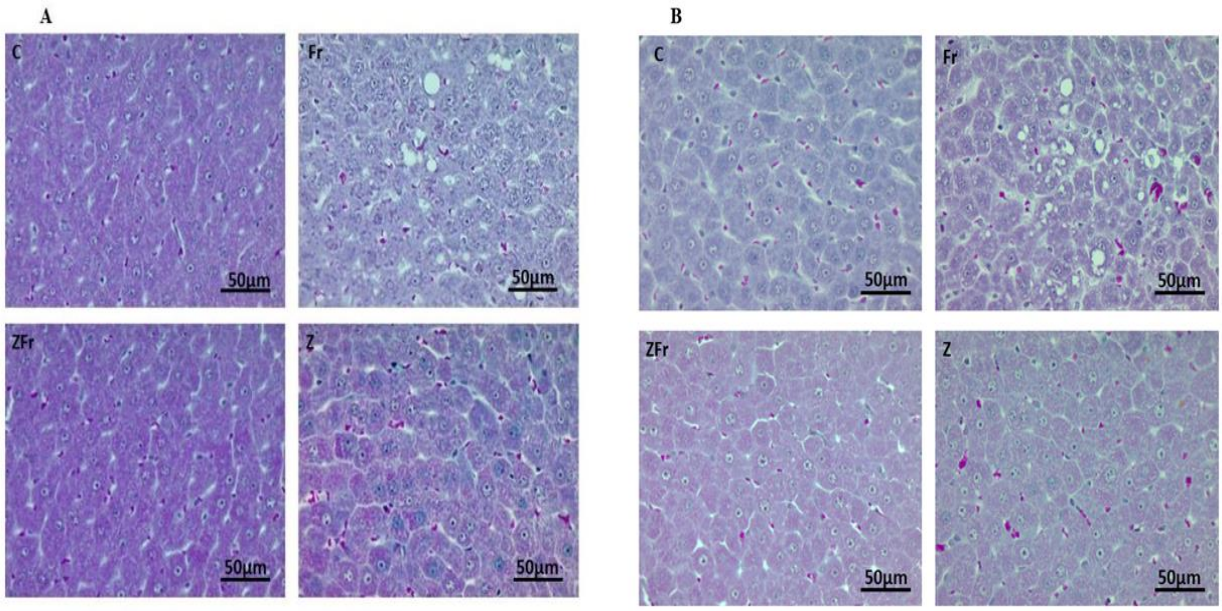

Figure 4.3: Representative photomicrographs of the male (Panel A) and female (panel B) rat liver sections stained with Masson's trichrome (x40).

C = 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 20; 9 males, 11 females); F r = 10 ml/kg body weight of a 20% fructose solution before weaning and free access to a 20% fructose solution after weaning (n = 20; 9 males, 11 females); ZFr = 10 ml/kg body weight of a 20% fructose solution combined with zingerone at 40 mg/kg body weight before weaning and free access to a 20% fructose solution after weaning (n = 21; 9 males, 12 females); Z = zingerone only at 40 mg/kg body weight in 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 18; 8 males, 10 females); there was no observable inflammation or fibrosis; scale bar = 50 µm.

4.4.3 Plasma alanine aminotransferase activity and concentrations of uric acid, thiobarbituric acid reactive substances and inflammatory markers

There were no differences in the activity of ALT [Males (P = 0.0633); Females (P = 0.2832)], concentrations of uric acid [Males (P = 0.5159); Females (P = 0.7666)], TBARS [Males (P = 0.9889); Females (P = 0.2282)], IL-6 [Males (P = 0.8979); Females (P = 0.9867)] and TNF-α [Males (P = 0.3966); Females (P = 0.4457)] across all the treatment groups (Table 4.3). Males had higher ALT activity than females [sex effect (P < 0.0001), treatment effect (P = 0.0114) and interaction effect (P = 0.0626); otherwise no sex difference (P > 0.05) was observed in the levels of uric acid, TBARS, IL-6 and TNF-α for sex, treatment and interaction effects.

Table 4.3: Effects of neonatally administered zingerone on the concentrations of alanine aminotransferase, uric acid, thiobarbituric acid reactive substances and levels of inflammatory markers of high-fructose diet fed rats

Parameter	Sex	C	Fr	ZFr	Z
ALT (U/L)	Male	75.00 ± 16.00#	66.00 ± 17.00#	53.00 ± 8.80#	81.00 ± 36.00#
	Female	53.00 ± 8.70	47.00 ± 8.00	50.00 ± 6.80	52.00 ± 7.90
Uric acid	Male	0.99 ± 0.56	1.30 ± 0.69	0.88 ± 0.42	1.10 ± 0.81
(mg/dL)	Female	0.76 ± 0.61	1.00 ± 0.55	0.92 ± 0.42	0.89 ± 0.66
TBARS	Male	22.00 ± 3.50	23.00 ± 3.80	22.00 ± 4.40	22.00 ± 3.60
(µM)	Female	22.00 ± 4.10	21.00 ± 4.30	24.00 ± 3.80	20.00 ± 4.60
IL-6	Male	60.74 ± 23.17	66.98 ± 17.72	60.57 ± 21.14	64.62 ± 21.93
(pg/mL)	Female	65.26 ± 18.97	66.12 ± 33.47	62.52 ± 19.16	64.97 ± 24.37
TNF-α	Male	125.99 ± 19.97	152.02 ± 42.08	140.76 ± 21.30	147.97 ± 45.44
(pg/mL)	Female	132.66 ± 50.00	143.85 ± 45.48	163.80 ± 54.31	149.32 ± 27.49

Data expressed as mean ± standard deviation. P > 0.05; # = male rats had higher ALT activity than their female counterparts at P < 0.0001; C = 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 20; 9 males, 11 females); Fr = 10 ml/kg body weight of a 20% fructose solution before weaning and free access to a 20% fructose solution after weaning (n = 20; 9 males, 11 females); ZFr = 10 ml/kg body weight of a 20% fructose solution combined with zingerone at 40 mg/kg body weight before weaning and free access to a 20% fructose solution after weaning (n = 21; 9 males, 12 females); Z = zingerone only at 40 mg/kg body weight in 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 18; 8 males, 10 females); ALT = alanine aminotransferase; TBARS = thiobarbituric acid reactive substances; IL-6 = interleukin 6; TNF-α = tumour necrosis factor alpha.

4.5 Discussion

The present study investigated the potential protective effects of neonatal administration of oral zingerone on long-term dietary fructose-induced fatty liver in Sprague-Dawley rats. Hepatic lipid accumulation and fatty infiltration of hepatocytes were observed when the male and female rats were terminated in early adulthood, following long-term administration of the 20% fructose solution. Zingerone administered orally to the neonatal rats protected them against high-fructose diet-induced increased hepatic lipid accretion and steatosis (both sexes). Thus, neonatal administration of oral zingerone was effective in attenuating the development of long-term fructose-induced NAFLD in both male and female rats. A sexually dimorphic response was observed only in plasma ALT activity where male rats had more activity of the enzyme than their female counterparts.

The mass of the liver relative to body mass is known as the hepato-somatic index [30], and it can be affected by nutritional status [31] and hepatic pathology including fatty liver diseases [30]. An increase in hepato-somatic index can result from a severe form of fatty liver disease including NASH [30], while a decrease in hepato-somatic index can result from undernutrition [31]. In the current study, we observed an increase in intrahepatic lipid accumulation in the high fructose-fed male and female rats, without affecting the hepato-somatic index. This finding is in agreement with Mamikutty et al.[12] who reported increased hepatic lipid deposition without an increase in liver weight in adult male Wistar rats that consumed 20% fructose solution for eight weeks. The hepato-somatic index that was not affected could be that the amount of lipid vacuoles in the hepatocytes of the study rats had not reached the threshold for inducing changes in the liver weight [12]. This finding indicated that the observed steatosis was not severe enough to cause NASH that is usually associated with increase in hepato-somatic index [30].

However, the neonatally administered zingerone conferred protection against the long-term dietary fructose-induced hepatic lipids accretion in both sexes. A previous study also reported the hepato-protective effect of zingerone in adult male rats, in which zingerone was administered orally at 100 mg/kg body weight for eight weeks and it prevented the intrahepatic lipid accumulation [24]. We have shown that by strategically targeting the neonatal period, a similar protective effect can be conferred. This beneficial effect of zingerone could have been due to its ability to enhance fatty acid oxidation via increase in hepatic PPAR activities thereby attenuating

hepatic lipogenesis [24,25]. In addition, zingerone could have prevented the fructose-induced hepatic lipid deposition (observed in this study) by attenuating hepatic *de novo* lipogenesis, which has been shown to be one of the deleterious effect of fructose on the liver [13]. Although excessive hepatic lipid content is of diagnostic importance for hepatic steatosis, the gold standard for diagnosis of NAFLD is histological assessment of hepatocytes [32].

Histologically, NAFLD primarily begins with fatty infiltration of hepatocytes when there are greater than 5% steatotic liver cells in the histological sections of the liver [5]. In the present study, we observed the presence of both micro-vesicular and macro-vesicular steatoses in both male and female rats that received the high fructose-diet only compared to the other groups. The observed fructose-induced steatosis in both sexes was prevented by neonatal administration of zingerone in this study, meaning that zingerone had programmed protection against the hepatic steatosis secondary to high-fructose diet consumption. The findings of this study are supported by those of a previous study, in older rats which reported that zingerone administered for eight weeks (at 100 mg/kg body weight) prevented the fructose-induced steatosis in adolescent male Wistar rats [24]. It is thus likely that zingerone protected against accumulation of lipids in the liver by down-regulating hepatic lipogenic genes and receptors including ChREBP, SREBP-1 and PPARα [24], since fructose has been reported to activate hepatic expression of these genes and receptors leading to increase hepatic lipid synthesis and storage [14]. However, the current study is limited in that we did not determine how these genes were expressed. Therefore, future studies on zingerone need to explore the molecular mechanisms involved in attenuating hepatic lipid accretion.

Additionally, zingerone has been reported to attenuate fructose-induced hepatic steatosis via decrease in hepatic pathways of fatty acids uptake, synthesis and secretion [24]. This effect of zingerone could have been responsible for the prevention of hepatic steatosis observed in this study.

Despite the presence of hepatic steatosis in the study rats, no evidence of inflammation or fibrosis was observed in both male and female rats in this study. Thus indicating that the rats in the present study did not develop advanced fatty liver disease which is typified by steatohepatitis and parenchymal tissue damage [24]. This has contrasted the findings of Narayanan and Jesudoss [24] who observed severe steatosis with associated inflammation and fibrosis following *ad libitum*

consumption of 20% fructose solution by adolescent male Wistar rats for eight weeks. The absence of inflammation and fibrosis in the histological samples of the study rats of both sexes could be explained based on the progressive nature of NAFLD which begins with simple steatosis before inflammation or fibrosis manifest [14]. Additionally, the age of rats at commencement of the study could also have had an impact as younger rats are less susceptible to developing fructose-induced metabolic disorders than older ones [33]. It is also noteworthy that only a small percentage (10-15%) of patients with steatosis develop inflammation and fibrosis (NASH) later in life [3]. Fatty liver could be associated with hepatocellular damage and elevated levels of the hepatic function marker, ALT [34].

We did not observe any significant differences in the blood levels of ALT in rats in the different groups of both sexes. The lack of impact of treatments on the ALT is supported by our histological findings that did not show evidence of inflammation, which is invariably associated with elevated ALT [12]. This lack of elevation in ALT secondary to a high fructose diet, was also reported by a previous study after *ad libitum* consumption of 20% fructose solution by adolescent male Wistar rats for 16 weeks [11]. Studies have shown that 25% of histologically confirmed NAFLD may exist without elevation of ALT [34]. Thus, this enzyme is mainly used for the assessment of hepatic biochemical function and hepatic damage, but not to predict NALFD [11].

Despite ALT activity not being affected by the interventions in both sexes, we observed that the activity of ALT was higher in male rats than females. It is not clear whether male sex hormones are responsible for the sex difference in the ALT activity [35]. However, since ALT is also produced by muscle cells [35], and male rats usually have higher muscle mass than females [36], we could speculate that the higher ALT activity observed in males could be due to their greater muscle mass when compared to females. Fructose-induced fatty liver is associated with elevated blood levels of uric acid [14] due to uncontrolled phosphorylation of fructose that converts ATP to adenosine monophosphate (AMP), which is eventually broken down to uric acid [13].

We observed that the plasma levels of uric acid were not different across the different treatments of both male and female rats in this study despite the presence of steatosis in the hepatocytes of fructose-treated groups. In rodents, uric acid is converted to allantoin by the enzyme uricase thereby decreasing circulating uric acid levels [37]. Humans normally do not have the enzyme uricase and are thus more prone to develop hyperuricaemia than rats when exposed to triggering

factors such as high fructose diets [37]. Fructose-induced hyperuricaemia is usually associated with severe forms of NAFLD than simple steatosis [14]. Additionally, Tapia et al.[38] demonstrated that severe metabolic derangement could be enhanced in rats when uricase is inhibited with oxonic acid. Thus, the findings of current study showing insignificant differences in uric acid concentrations could be explained by the effect of uricase in rats and the moderate NAFLD observed. Moreover, it has also been shown that fructose-induced fatty liver can develop with or without hyperuricaemia [39]. Fructose-induced fatty liver can lead to oxidative stress secondary to lipid peroxidation [13], and subsequent hepatic inflammation [40].

Although zingerone has been reported to prevent hepatic damage by inhibiting the generation of free radicals and downregulating inflammatory cytokines of hepatic inflammation [23–25], the treatments had no effect on the levels of TBARS, IL-6 and TNF-α in male and female rats in the present study. Fakhoury-Sayegh et al.[11] also reported similar findings when 20% fructose solution was consumed *ad libitum* by adolescent male Wistar rats for 16 weeks. These findings suggested that the interventions did not cause an overt pro-inflammatory state, a finding that can be reinforced by histological features of the rats' hepatocytes as there were no evident inflammatory foci, further confirming the presence of a non-severe form of the disease that had not progressed to NASH.

4.6 Conclusion

Neonatal administration of zingerone orally to Sprague-Dawley rats prevented the long-term fructose-induced fatty liver development in both sexes. A common mantra amongst health care professionals is that 'prevention is better than cure'. Targeting this critical period of developmental plasticity, with a naturally derived phytochemical may provide an alternative prophylaxis for NAFLD and hence reduce the burden of this condition on health care facilities globally. Future studies will be directed towards understanding the mechanisms of action of zingerone that resulted in these long-term protective effectives and the possible transgenerational benefits of the interventions. Additionally, the interaction of fructose with micronutrients such as copper will also be considered in future studies since fructose has been shown to alter the utilization of copper [41].

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The next chapter, which is a manuscript currently under review, describes the potential protective effect of oral zingerone administration to neonatal rats on the development of fructose-induced nephropathy.

CHAPTER FIVE - Submitted manuscript currently under review

Neonatal administration of zingerone prevents the subsequent development of high dietary fructose-induced early features of nephropathy in rats

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(2021)

Animal Physiology and Animal Nutrition (Manuscript ID: JAPAN-Apr-21-285)

5.1 Abstract

Excessive consumption of fructose-rich diets in early life stages increases the risk for developing nephropathy in adulthood, necessitating dialysis and kidney transplants. To investigate the potential preventive effects of neonatally administered zingerone on the development of fructose-induced nephropathy, four-day old suckling rat pups (males = 35; females = 44) were orally gavaged [10 ml/kg body weight (bwt)] with: distilled water (Con), 20% fructose solution (Fru), Fru + 40 mg/kg bwt zingerone in distilled water (ZFru), or 40 mg/kg bwt of zingerone (Zgr) for 14 days. Thereafter, Con and Zgr groups continued on plain drinking water while Fru and ZFru groups drank 20% fructose solution *ad libitum* for 10 weeks, in addition to commercial rat feed. The fructose only administered group (Fru) had significantly [$P = 0.0092$ (males); $P < 0.0003$ (females)] increased KIM-1 levels, and decreased urinary space [$P < 0.0076$ (males); $P < 0.0067$ (females)] compared to the controls (Con). Neonatally administered zingerone prevented the fructose-induced increase in KIM-1 levels [$P = 0.9996$ (males); $P = 0.3581$ (females)], and fructose-induced reduction in the urinary space [$P = 0.9878$ (males); $P = 0.9696$ (females)] when ZFru was compared with the controls. Kidney masses, malondialdehyde levels, corpuscular and glomerular tuft areas, and glomerular density were similar ($P > 0.05$) across the groups. Zingerone administration neonatally is a potential prophylaxis for long-term high-fructose diet-induced nephropathy.

Keywords: Fructose, nephropathy, zingerone, programming, neonates, rats

5.2 Introduction

The global prevalence of chronic kidney disease is rapidly increasing, affecting between 8 and 18 percent of the world population [1], and is projected to be among the five top causes of death by 2040 [2]. Chronic kidney disease is invariably associated with end stage renal disease and cardiovascular mortality [2]. Early postnatal life events can alter the structure and/or functions of the developing kidney leading to the development of kidney disease later in life [3]. Physiologically, the developing kidney, starts functioning only after birth and continues to develop and mature up to postnatal day 14 in rats [3,4]. Thus, adverse events during the early lactation period may adversely affect nephrogenesis leading to epigenetic changes that can programme for chronic kidney disease in adult life [3]. Early life malnutrition affects the metabolic

function of kidneys [5] leading to acute and reversible kidney diseases, which may progress to long-term irreversible chronic kidney diseases [3].

The rise in fructose consumption over the last century has paralleled the rising prevalence of kidney diseases, leading to the hypothesis that excessive fructose might be a causal factor for the development of chronic kidney disease [6]. Fructose is metabolized in the kidney and then phosphorylated by ketohexokinase which is heavily expressed in the proximal tubule of the kidney nephron [7]. The phosphorylation of fructose causes adenosine triphosphate (ATP) depletion and subsequent generation of oxidative stress secondary to lipid peroxidation [8,9], thereby damaging the renal parenchyma [6] and contributing significantly to the progression of nephropathy [10]. Nakayama et al. [7] previously reported that a high-fructose diet accelerated chronic renal disease via an increase in tubulointerstitial injury and glomerulosclerosis in rats.

The gold standard for early diagnosis of renal diseases is kidney biopsy, which can also be used to determine the patterns and progression of the disease [11]. Specific histopathology and morphometric changes such as alterations in urinary spaces, glomerular tuft and renal corpuscle areas are subjects of the kidney histological assessment [4,12]. However, high costs and the invasive nature of kidney biopsy make it unaffordable to most patients, and thus it is not routinely performed in patients with kidney diseases [13]. It has been shown that metabolites measured in plasma or renal tissues can be used as kidney disease-relevant biomarkers for renal disease pathogenesis, progression, prediction, and diagnosis [14]. Kidney injury molecule 1 (KIM-1) and tissue malondialdehyde (MDA), have both been shown to be affected by high-fructose diets [14] and therefore are potential viable markers of renal disease.

Kidney injury molecule (KIM-1) is a protein produced during toxic insults on the kidney and was observed as a sensitive and specific biomarker for early prediction of acute and chronic kidney damage in experimental animals [14,15]. Whilst KIM-1 is a potent target for therapeutic intervention of nephropathy in experimental models [15], MDA is a product of lipid peroxidation and thus a marker of oxidative stress [15].

Despite no specifically approved drug treatment for kidney diseases, lifestyle modifications inclusive of regular exercise and good dietary habits are the first approaches in the early management of diet-induced nephropathy [16]. The use of synthetic pharmacological agents (both specific and non-specific) in the management of kidney diseases is limited to symptomatic

treatment, and these agents are usually monotherapeutic and associated with adverse effects [2]. Additionally, the synthetic pharmacological agents may only slow the progression of nephropathy but do not completely stop it [2]. Advanced chronic kidney diseases pose a great economic burden on patients and government health care as dialysis and kidney transplants are the only alternative treatment options for late stages of the disease [13].

Globally, communities are increasingly opting for natural products including phytochemicals for their healthcare needs [15,17] in the belief that they provide a holistic, multi-spectrum nutraceutical value and are easily accessible [15]. Previous studies have used phytochemicals during the immediate postnatal period to programme for long-term metabolic health outcomes in fructose-fed rats. The development of hepatic lipid accumulation was prevented in adult rats fed high-fructose diets following postnatal administration of oleanolic acid [18] and S-allyl cysteine [19]. In the current study, we investigated whether zingerone could be used to prevent fructose-induced nephropathy.

Zingerone is a phenolic alkanone found in the ginger (*Zingiber officinale*) plant [20]. Previous studies have documented the antioxidant, anti-inflammatory and nephroprotective properties of zingerone in adult rats [15] and mice [10] experimental models of diabetic nephropathy. The safety of zingerone has also been established with LD_{50} of 2580 mg/kg in rats [15]. Given the high burden that management of chronic kidney disease places on healthcare systems and the absence of specific treatments for the disease, there is need to develop alternative strategic prophylactic interventions. The biological properties of zingerone made it a suitable candidate phytochemical in the current study for administration during the neonatal period to explore its potential to protect against long-term development of nephropathy secondary to high-fructose diets. In that regard, due to close physiological relationship between rats and humans [21], using a similar model of exploring zingerone neonatally could be translated in humans to manage diet-induced nephropathy; a strategy that could replace the use of animal models in exploring the health beneficial effects of zingerone.

5.3 Experimental Methods

5.3.1 Experimental setting and procedures

The study was approved by the Animal Research Ethics Committee (AREC) of the University of Witwatersrand (Clearance No: 2017/010/71/B). It was conducted at the Central Animal Service

(CAS) of the University of Witwatersrand, Johannesburg, South Africa, using the internationally accepted principles for laboratory animal use and care [South African National Standard (SANS 10386:2008) and Animals Protection Act, 1962: Act No. 71]. The protocol for the study was submitted to the AREC and has been archived there. The Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines were adopted while compiling the manuscript.

The data reported in this manuscript are part of the larger data set of the study. The data were divided into subsets because the study was conducted in three stages. The data collected in stages 1 and 2 are not included in this manuscript. During stage 1, the potential preventive effects of neonatal zingerone intake on the development of fructose-induced metabolic derangements in male and female rats was investigated, and the data collected are published in the Journal of Developmental Origins of Health and Disease [22]. In stage 2, we investigated the potential preventive effects of neonatally administered zingerone on the development of fructose-induced NAFLD in male and female rats, and the data collected are published in the Journal of Medicinal Food [23]. The third (current) stage explored the potential preventive effects of neonatally administered zingerone on the development of fructose-induced nephropathy in male and female rats.

The study consisted of two stages: Pre-weaning (postnatal day 8-21) and post-weaning (postnatal day 22-91). Seven nulliparous dams (Sprague-Dawley rats), each with a litter consisting of 8-12 four-day-old suckling male and female pups (weighing 9.7 ± 1.1 g) were used in this study. The rats were obtained from the Central Animal Service, University of the Witwatersrand. The rat mating pairs were put together by animal attendants who were blinded to the study design. They then selected dams with the appropriate litter sizes and these were then availed to the researcher. On postnatal day 8 (after the pups were acclimatized to the environmental conditions for 4 days), the pups from each dam (n = 79; males = 35, females = 44) were randomly allocated to four treatment groups. The researcher created the allocation sequence. Rat pups from each dam were first stratified by sex. Thereafter they were placed in a cage and as taken out individually, they were proportionally and sequentially allocated to each treatment group, marked on their tails with non-toxic ink for identification and returned to their dams. Resultantly, pups from each litter were represented in each one of four treatment groups. **Group 1** (Negative control) received 10 ml/kg body weight (bwt) of distilled water gavaged orally before weaning and *ad libitum* access to plain tap water to drink after weaning (n = 20; males = 9, females =

11); **Group 2** received 10 ml/kg bwt of a 20% fructose solution gavaged orally before weaning and *ad libitum* access to a 20% fructose solution to drink after weaning (n = 20; males = 9, females = 11) to induce metabolic dysfunction; **Group 3** received 10 ml/kg bwt of a 20% fructose solution + zingerone at 40 mg/kg bwt gavaged orally before weaning (to programme for protection against long-term high fructose consumption) and *ad libitum* access to a 20% fructose solution to drink after weaning (n = 21; males = 9, females = 12); **Group 4** received 40 mg/kg bwt of zingerone dissolved in 10 ml/kg bwt of distilled water gavaged orally before weaning and *ad libitum* access to plain tap water to drink after weaning (n = 18; males = 8, females = 10) to assess the effects of neonatal intake of zingerone alone on renal metabolic health of the animals.

The dams with their respective litters were housed in Perspex cages containing wood shavings as bedding. The room temperature was maintained at 25 ± 1 °C and a 12-h light and dark cycle with lights off from 7 p.m. to 7 a.m. The dams were supplied with commercially formulated standard rat chow (LabChef Research Nutrition, (Pty) Ltd, Stellenbosch, South Africa) and *ad libitum* water access throughout the suckling period. The pups were allowed to freely nurse from their respective dams until weaning on postnatal day 21. The dams were returned to stock after weaning of their pups. The weaned rats were then housed individually in Perspex cages having the same environmental conditions described earlier.

The fructose solution (Natures Choice, South Africa) was constituted to 20% by adding 20 g of fructose into 100 ml of water [24], while food grade zingerone (W312401-1KG) was purchased from Sigma-Aldrich (USA), and it is ≥ 98% natural. The standard rat chow was provided by the LabChef Research Nutrition (Pty) Ltd, Stellenbosch, South Africa. The nutritional compositions of fructose and standard rat chow are shown in Table 5.1.

The rats were weighed daily during the pre-weaning stage, twice a week during the post-weaning stage and prior to termination by individually placing the rats in a pre-weighed cage on an electronic balance (Snowrex Electronic Scale, Clover Scales, Johannesburg, South Africa). This was to monitor the animals' growth and adjust treatment dosages.

Table 5.1: Nutritional composition of fructose (*A*) and standard rat chow (*B*)

A

Fructose Nutritional analysis	Composition (per 100g)
Energy	1680 KJ
Carbohydrates	99.8 g
Protein	0 g
Fats	0 g
Fibre	0 g
Sodium	0.2 mg

B

Rat Chow Ingredients	Composition
	g/kg
Protein	220
Moisture	100
Oils and fats	50
Linoleic acid	12
Fibre	40
Ash	70
Calcium	12
Phosphorous	7.5
Vitamin E	100
	IU/kg
Vitamin A	16 000
Vitamin D	2000

5.3.2 Procedures at termination

After the 12-week intervention period, the rats were fasted from solid feed but provided plain drinking water overnight for 12 hours. Terminal body mass was then determined with an electronic balance (Snowrex Electronic Scale, Clover Scales, Johannesburg, South Africa) and the rats were then euthanised with sodium pentobarbitone (Centaur Laboratories, Johannesburg, South Africa) at 150 mg/kg body weight intraperitoneally.

After euthanasia, a ventral midline incision was made through the thorax and abdomen; syringes with attached needles were used to withdraw blood samples from the heart. The blood samples were then transferred into heparinized tubes (BD Vacutainer, Plymouth, UK) and centrifuged (Rotofix 32A, Hettich Zentrifugen, Germany) at 3700 g for 15 minutes at 20 °C. The plasma was harvested and stored at -20 °C for determination of KIM-1. The kidneys were carefully dissected out from the abdominal cavity, perirenal fat removed, and weighed using a Presica 310M balance (Precision Instruments, Johannesburg, South Africa)], and then the weight of both kidneys relative to body mass computed:

Relative kidney weight (%) = [mass of kidneys (g) / terminal body mass (g)] × 100

The right kidney was preserved in 10% phosphate buffered formalin for histological analysis, while the left kidney was preserved at -20 °C for tissue MDA determination.

5.3.3 Determination of plasma KIM-1 and kidney tissue MDA

Plasma KIM-1 was determined using a rat-KIM-1 Enzyme Linked Immuno-Sorbent Assay kit (Elabscience Biotechnology Co., Ltd, Wuhan, Hubei, China) according to manufacturer's instructions.

To determine kidney MDA, the frozen preserved kidneys were allowed to thaw at room temperature, and then homogenised in phosphate buffered saline with a glass homogeniser on ice. The cells were further fragmented by sonicating the suspension with an ultrasonic cell disrupter. The homogenates were then centrifuged for 5 minutes at 5000×g to obtain the supernatant. The tissue homogenate MDA was then determined using rat-MDA Enzyme Linked Immuno-Sorbent Assay kit (Elabscience Biotechnology Co., Ltd, Wuhan, Hubei, China) according to manufacturer's instructions.

5.3.4 Histological assessment of the kidney

The kidney samples that were preserved in 10% phosphate buffered formalin were processed using an automatic tissue processor (Microm STP 120, Thermo Scientific, MA, USA), embedded in paraffin wax, sectioned at 5µm and then stained with haematoxylin and eosin (H&E) or Masson's trichrome (MT). The slides were viewed under a light microscope [Axioskop 2 microscope; (Leica Biosystems, USA)] mounted to an Axiocam HRc2 camera, which uses ZEISS ZEN microscope software for image capture. Measurements were taken on the photomicrographs using the Zen lite analysis software. The images at 40x magnification from the H&E stained slides were used to assess corpuscular and glomerular tuft areas under 40x magnification, and glomerular density under 10x magnification.

Urinary space area was computed using the formula [25]:

Urinary space area (μm^2) = Renal corpuscular area (μm^2) – Glomerular tuft area (μm^2)

Glomerular density was computed using the following formula [26]:

Glomerular density ($N/\mu m^2$) = Number of renal corpuscle per section (N) ÷ Total area of the section (μm^2)

The images at 40x magnification of the MT stained slides were used to assess collagen deposition and fibrosis.

5.3.5 Statistical analysis

Multiple-group data were analysed using Graphpad Prism 8 software (Graph-pad Software Inc., San Diego, USA), by one-way analysis of variance (ANOVA), and expressed as mean ± standard deviation (SD); followed by multiple-comparisons Tukey *post hoc* tests. Sex variations were analysed using two-way ANOVA. Statistical significance was considered when $P < 0.05$.

We used a previous study conducted in our research laboratory to calculate the sample size (8-12 rats per group) [19] using the formula [27]:

Sample size = $2\ SD^2\ (1.96 + 0.842)^2/d^2$

Where SD = standard deviation from previous study; 1.96 = type 1 error of 5%; 0.842 = at 80% power; d = difference between mean values.

5.4 Results

5.4.1 Kidney mass

The absolute [$P = 0.4644$ (Males); $P = 0.0897$ (Females); ANOVA] and relative [$P = 0.5405$ (Males); $P = 0.0759$ (Females); ANOVA] masses of the kidneys were not different across the different treatments in both sexes (Table 5.2). However, male rats had significantly greater absolute kidney mass than females ($P < 0.0001$ for sex effect, $P = 0.1740$ for treatment effect and $P = 0.7384$ for interaction effect; ANOVA), while female rats had significantly greater relative kidney masses than the males ($P = 0.0117$ for sex effect, $P = 0.0842$ for treatment effect and $P = 0.6807$ for interaction effect; ANOVA).

Table 5.2: Effects of neonatally administered zingerone on the absolute and relative kidney masses of high-fructose diet fed rats

Parameter	**Sex**	**Con**		**Fru**		**ZFru**		**Zgr**			
		Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*95% CI*	*P*
Kidney mass (g)	Male	2.40$^{\alpha}$	0.21	2.40$^{\alpha}$	0.24	2.30$^{\alpha}$	0.19	2.30$^{\alpha}$	0.18	-0.24, 0.29	0.4644
	Female	1.60	0.11	1.60	0.05	1.50	0.12	1.60	0.18	-0.17, 0.11	0.0897
Relative kidney mass (% bwt)	Male	0.63	0.03	0.61	0.03	0.61	0.03	0.62	0.02	-0.020, 0.056	0.5405
	Female	0.65$^{\beta}$	0.06	0.63$^{\beta}$	0.04	0.62$^{\beta}$	0.03	0.66$^{\beta}$	0.05	-0.029, 0.071	0.0759

Data expressed as mean and standard deviation (SD) using analysis of variance (ANOVA); CI = confidence interval; P = predictive value; Con = 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n =20; 9 males, 11 females); Fru = 10 ml/kg body weight of a 20% fructose solution before weaning and free access to a 20% fructose solution after weaning (n = 20; 9 males, 11 females); ZFru = 10 ml/kg body weight of a 20% fructose solution combined with zingerone at 40 mg/kg body weight before weaning and free access to a 20% fructose solution after weaning (n = 21; 9 males, 12 females); Zgr = zingerone only at 40 mg/kg body weight in 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 18; 8 males, 10 females); % bwt = percentage body weight; $^{\alpha}$ = male rats had higher absolute kidney masses than their female counterparts at P < 0.0001 and 95% CI 0.70 to 0.85; $^{\beta}$ = female rats had higher relative kidney masses than their male counterparts at P = 0.0117 and 95% CI -0.040 to -0.0052.

5.4.2 Plasma KIM-1 and kidney tissue MDA

The fructose only administered group (Fru) had significantly [$P = 0.0092$ (Males); $P < 0.0003$ (Females); ANOVA] increased levels of KIM-1 compared to the controls (Con) in both sexes (Table 5.3). Neonatally administered zingerone prevented the fructose-induced increase in the levels of KIM-1 such that rats administered combined fructose and zingerone (ZFru) had similar [$P = 0.9996$ (Males); $P = 0.3581$ (Females); ANOVA] concentration of KIM-1 when compared with the controls in both sexes. There were no differences in the levels of kidney tissue MDA [$P = 0.5000$ (Males); $P = 0.2123$ (Females); ANOVA] across all the treatment groups.

No sex difference was observed in the plasma levels of KIM-1 [$P = 0.2858$ (sex effect); $P = 0.5201$ (interaction effect); ANOVA] and tissue MDA [$P = 0.1412$ (sex effect); $P = 0.1966$ (interaction effect); $P = 0.6772$ (treatment effect); ANOVA].

Table 5.3: Effects of neonatally administered zingerone on the plasma levels of KIM-1 and kidney tissue MDA of high-fructose diet fed rats

Parameter	**Sex**	**Con**		**Fru**		**ZFru**		**Zgr**			
		Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*95% CI*	*P*
KIM-1 (pg/mL)	Male	61.00^{a}	30.00	104.00^{b}	26.00	62.00^{a}	20.00	70.00^{a}	31.00	-77, -8.9	0.0092
	Female	50.00^{a}	22.00	97.00^{b}	34.00	67.00^{a}	17.00	55.00^{a}	19.00	-74, -19	0.0003
MDA (ng/mL)	Male	61.00	30.00	78.00	22.00	66.00	28.00	66.00	17.00	-14, 49	0.5000
	Female	56.00	18.00	67.00	18.00	66.00	21.00	52.00	22.00	-33, 12	0.2123

Data expressed as mean and standard deviation (SD) using analysis of variance (ANOVA); ^{ab} = within row means with different superscript letters significantly different at P < 0.05; CI = confidence interval; P = predictive value; KIM-1 = kidney injury molecule 1; MDA = malondialdehyde; Con = 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 20; 9 males, 11 females); Fru = 10 ml/kg body weight of a 20% fructose solution before weaning and free access to a 20% fructose solution after weaning (n = 20; 9 males, 11 females); ZFru = 10 ml/kg body weight of a 20% fructose solution combined with zingerone at 40 mg/kg body weight before weaning and free access to a 20% fructose solution after weaning (n = 21; 9 males, 12 females); Zgr = zingerone only at 40 mg/kg body weight in 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 18; 8 males, 10 females).

5.4.3 Histomorphometry of the kidney

The fructose only administered group (Fru) had significantly [$P < 0.0076$ (Males); $P < 0.0067$ (Females); ANOVA] decreased urinary space area compared to the controls (Con) in both sexes (Figure 5.1 and Table 5.4). Neonatally administered zingerone prevented the fructose-induced decrease in the urinary space area such that rats administered combined fructose and zingerone (ZFru) had similar [$P = 0.9878$ (Males); $P = 0.9696$ (Females); ANOVA] urinary space area when compared with the controls in both sexes. This demonstrated that zingerone prevented the development of early histological signs of nephropathy induced by high-fructose diet in the study rats. There were no differences in the renal corpuscular area [$P = 0.1393$ (Males); $P = 0.8579$ (Females); ANOVA], glomerular tuft area [$P = 0.3252$ (Males); $P = 0.6692$ (Females); ANOVA] and glomerular density [$P = 0.4847$ (Males); $P = 0.7067$ (Females); ANOVA] across all the treatment groups. No evidence of tubular damage was observed in both sexes.

No sex difference was observed in the corpuscular area [$P = 0.9976$ (sex effect); $P = 0.4479$ (interaction effect); $P = 0.2095$ (treatment effect); ANOVA], glomerular tuft area [$P = 0.9664$ (sex effect); $P = 0.5677$ (interaction effect); $P = 0.3716$ (treatment effect); ANOVA], urinary space area [$P = 0.8691$ (sex effect); $P = 0.4961$ (interaction effect); ANOVA] and glomerular density [$P = 0.9778$ (sex effect); $P = 0.4490$ (interaction effect); $P = 0.7476$ (treatment effect); ANOVA].

For the Masson's trichrome histological sections (MT stain), there was no observable collagen deposition or fibrosis across the different treatment groups in both sexes [Figure 5.2A (Males); Figure 5.2B (Females)].

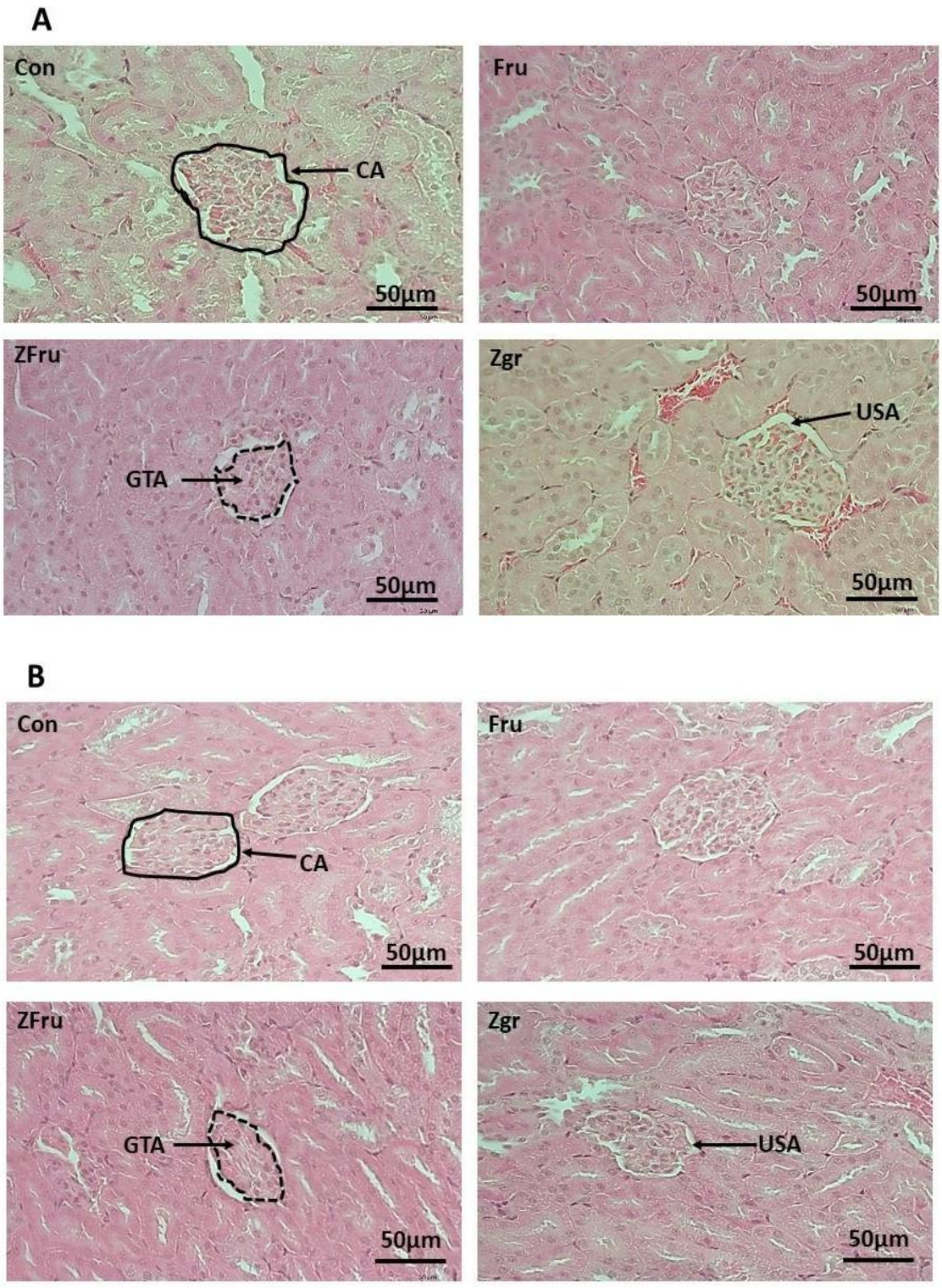

Figure 5.1: Representative photomicrographs of the male (Panel A) and female (Panel B) rat kidney sections stained with Haematoxylin and Eosin (x40).

Con = 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 20; 9 males, 11 females); Fru = 10 ml/kg body weight of a 20% fructose solution before weaning and free access to a 20% fructose solution after weaning (n = 20; 9 males, 11 females); ZFru = 10 ml/kg body weight of a 20% fructose solution combined with zingerone at 40 mg/kg body weight before weaning and free access to a 20% fructose solution after weaning (n = 21; 9 males, 12 females); Zgr = zingerone only at 40 mg/kg body weight in 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 18; 8 males, 10 females); CA = corpuscular area; GTA = glomerular tuft area; USA = urinary space area; scale bar = 50 µm.

Table 5.4: Effects of neonatally administered zingerone on kidney morphometry of the fructose-fed rats.

Parameter	**Sex**	**Con**		**Fru**		**ZFru**		**Zgr**			
		Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*95% CI*	*P*
Corpuscular area (μm^2)	Male	15370.00	4151.00	15016.00	3877.00	16151.00	4584.00	19928.00	5384.00	-5838, 6544	0.1393
	Female	15702.00	4000.00	16440.00	3679.00	17339.00	4316.00	16971.00	3944.00	-6187, 4710	0.8579
Glomerular tuft area (μm^2)	Male	12614.00	3521.00	14012.00	3553.00	13557.00	4433.00	16247.00	4267.00	-6810, 4015	0.3252
	Female	12765.00	3932.00	15046.00	3636.00	14596.00	3992.00	13857.00	3996.00	-7595, 3032	0.6292
Urinary space area (μm^2)	Male	2755.00^a	1077.00	1005.00^b	500.90	2594.00^a	620.30	3681.00^a	1465.00	395.6, 3106	0.0076
	Female	2937.00^a	815.90	1394.00^b	491.20	2744.00^a	939.20	3114.00^a	1090.00	364.9, 2721	0.0067
G.D. ($N/\mu m^2$; $\times 10^{-6}$)	Male	3.36	7.80	3.86	7.09	3.23	1.13	3.41	3.41	-1.67, 6.65	0.4847
	Female	3.23	8.97	3.38	8.12	3.66	1.04	3.63	3.63	-1.33, 1.03	0.7067

Data expressed as mean and standard deviation (SD) using analysis of variance (ANOVA); ^{ab} = within row means with different superscript letters significantly different at P < 0.05; CI = confidence interval; P = predictive value; Con = 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 20; 9 males, 11 females); Fru = 10 ml/kg body weight of a 20% fructose solution before weaning and free access to a 20% fructose solution after weaning (n = 20; 9 males, 11 females); ZFru = 10 ml/kg body weight of a 20% fructose solution combined with zingerone at 40 mg/kg body weight before weaning and free access to a 20% fructose solution after weaning (n = 21; 9 males, 12 females); Zgr = zingerone only at 40 mg/kg body weight in 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 18; 8 males, 10 females); G.D.= glomerular density.

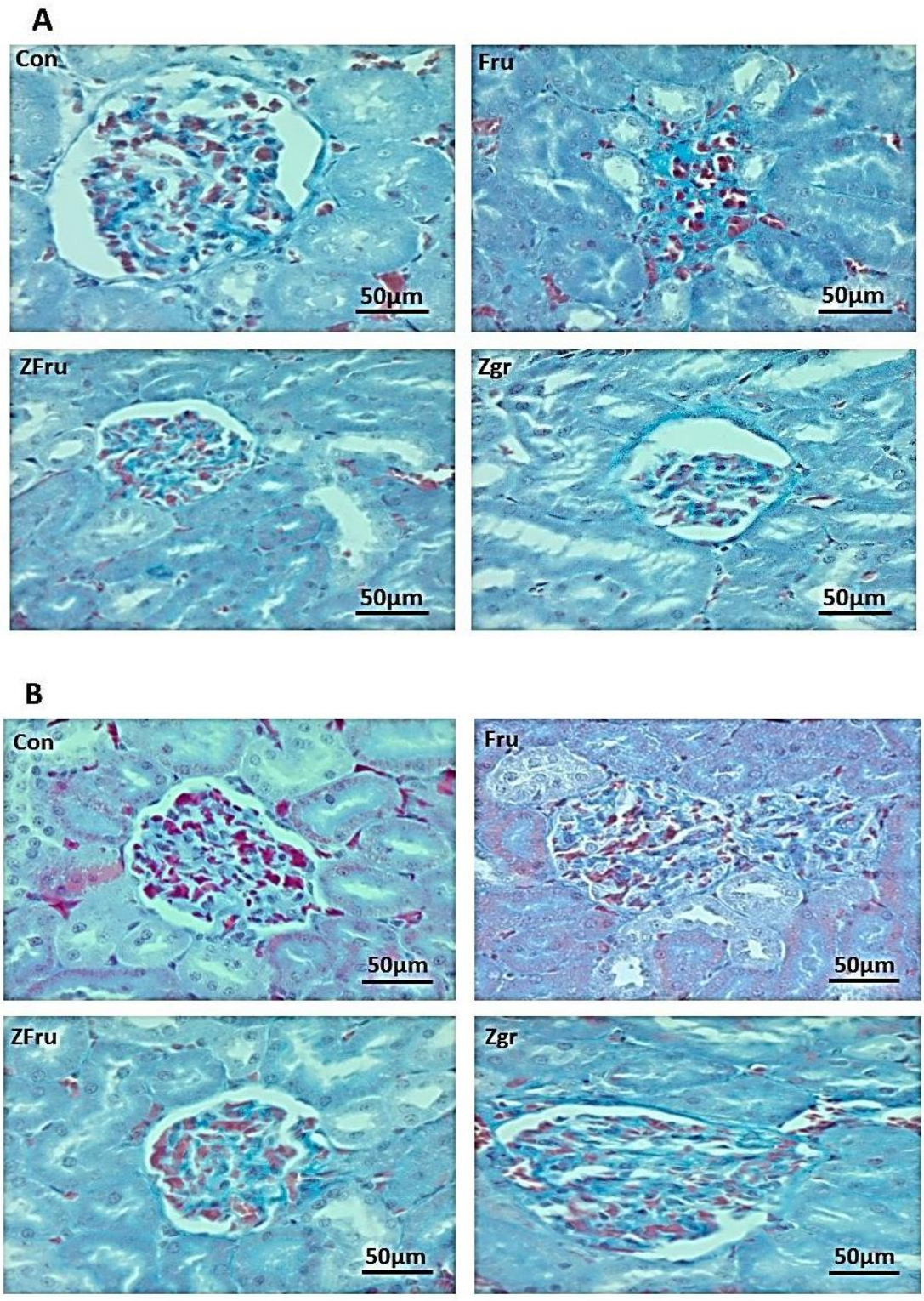

Figure 5.2: Representative photomicrographs of the male (Panel A) and female (Panel B) rat kidney sections stained with Masson's trichrome (x40).

Con = 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 20; 9 males, 11 females); Fru = 10 ml/kg body weight of a 20% fructose solution before weaning and free access to a 20% fructose solution after weaning (n = 20; 9 males, 11 females); ZFru = 10 ml/kg body weight of a 20% fructose solution combined with zingerone at 40 mg/kg body weight before weaning and free access to a 20% fructose solution after weaning (n = 21; 9 males, 12 females); Zgr = zingerone only at 40 mg/kg body weight in 10 ml/kg body weight of distilled water before weaning and free access to plain tap water after weaning (n = 18; 8 males, 10 females); scale bar = 50 µm.

5.5 Discussion

We investigated the potential protective effects of neonatal administration of oral zingerone on long-term dietary fructose-induced nephropathy later in adulthood. Increased levels of plasma KIM-1 and reduced urinary space area were observed in the adult male and female rats following long-term administration of the 20% fructose solution. Zingerone administered orally to the neonatal rats protected them against high-fructose diet-induced increased in KIM-1 and the reduced urinary space area later in adulthood in both sexes. Thus, neonatal administration of oral zingerone attenuated the development of long-term fructose-induced nephropathy in both male and female rats in adult life. A sexually dimorphic response was observed only in kidney masses, where the absolute kidney masses were higher in males and the relative kidney masses were higher in females.

The weight of a kidney is an important parameter in evaluating renal tissue hypertrophy that may be associated with nephropathy [10]. Additionally, changes in organ weights are used in toxicology to determine whether a particular substance has adverse effects which may cause atrophy [28]. The current study did not note any differences in the absolute weight of the kidneys of the study rats within both sexes, implying that the interventions did not produce gross effects on the kidneys. The fact that male rats had greater absolute kidney weights than females could be attributed to inherent body sizes of the male rats compared for females [29], due to greater muscle mass [30] and organ weight [31] in males than female rats.

However, when scaled relative to body mass it was observed that the females had relatively heavier kidneys. This may be attributed to the fact that the kidneys stop growing at a particular age [4], however in male rats the body mass increase is relatively greater than female rats [29]. The observed increase in relative kidney weight in female rats compared to their male counterparts could also probably be attributed to fewer nephron number in female rat kidneys when compared to male kidneys, thereby making the kidney index higher in female rats [29].

Renal function can be assessed using different parameters including glomerular filtration rate, blood urea and creatinine [32]. Measurement of glomerular filtration rate and blood urea and creatinine levels is commonly employed to assess renal function; however, these measurements are normally diagnostic in cases of severe renal injury and have limitations in identifying and monitoring kidney diseases [33]. It is therefore important to use more sensitive and direct

assessments. Metabolomics show that plasma [15] and urinary [34] KIM-1 is affected in the early stages of nephropathy.

In the current study, we observed an increase in the plasma levels of KIM-1 in both male and female rats that received the long-term high fructose-diet only compared to the other groups. The fructose-induced increase in KIM-1 was prevented by neonatal administration of zingerone, meaning that neonatal zingerone had programmed protection against the renal injury secondary to high-fructose diet consumption. The finding of this study expands on that of a previous study that reported a decrease in KIM-1 following administration of 50 mg/kg body weight of zingerone to streptozotocin-induced diabetic adult male Wistar rats for 16 weeks [15]. Thus, we have shown a prophylactic effect while Rehman et al. [15] showed a therapeutic effect of zingerone. This beneficial effect of zingerone has been partially attributed to its antioxidant properties including suppression of MDA production, which is a lipid peroxidation product [35].

An increase in MDA level is an indicator of nephropathy associated oxidative stress [15]. In the current study, neither administration of fructose nor that of zingerone had any effects on the kidney tissue level of MDA in the study rats in both sexes. This is in contrast to a previous report that demonstrated zingerone to inhibit oxidative stress via decreasing MDA in diabetic mice [10]. The disparity could be that the high-fructose diet did not cause severe kidney damage (evident on histology) in the study rats that were not diabetic-induced.

Assessment of kidney histopathology is considered as the gold standard in the clinical diagnosis of nephropathy [36]. Additionally, histological lesions of kidneys may develop without derangement of metabolomics in nephropathy [11]. In the present study, we observed a reduction in the urinary space area in the kidney sections of both male and female rats that received high fructose-diet only compared to the other groups, without change in the corpuscular area, glomerular tuft area and glomerular density. Decreased urinary space area is an early histologic feature of nephropathy that can lead to reduced glomerular filtration capacity and eventual kidney failure [10]. The observed fructose-induced histological lesions in both sexes were prevented by neonatal administration of zingerone in this study, implying that zingerone had programmed protection against the early histological features of nephropathy secondary to high-fructose diet consumption. The observed histological outcomes in the current study require

further investigations involving bio-histochemical assessments to establish the fundamental physiological mechanisms.

Whilst corpuscular area, tuft area and glomerular density are important parameters in evaluating nephropathies [4], these parameters were not affected in this study. This implied that the rats on high- fructose diet had developed only early features of the disease since these parameters are usually altered in advanced renal disease [12]. The absence of glomerular, tubular or interstitial lesions (particularly fibrosis) in the histological sections of the fructose-fed rats despite elevated KIM-1 further supported this assertion. That the rats did not develop advanced nephropathy, is not surprising since a significant proportion of individuals do have early histological features of nephropathy without affecting the tubules and surrounding interstitium [11].

5.6 Conclusion

Early postnatal administration of zingerone together with fructose to rats prevented the long-term fructose-induced early nephropathy as measured with the early renal injury marker KIM-1 and confirmed by histological assessment in adulthood in both sexes. These findings present a potential alternative strategic prophylactic intervention to reduce the global burden of nephropathy in humans by targeting a critical period of developmental plasticity with this naturally derived phytochemical. This study has also shown that zingerone could be safe for administration in the neonatal period, despite reports that neonatal treatment with phytochemicals could cause toxicity [37] as most physiological systems are not fully developed [38]. Future studies will be directed towards understanding the mechanisms of action of zingerone that resulted in these long-term reno-protective effects.

5.7 References

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CHAPTER SIX - Effects of neonatally administered zingerone on general health status of rats fed a high-fructose diet

6.1 Introduction

The global prevalence of metabolic disorders is projected to be 20-25% in the adult population [1], and 3.3% in the paediatric age group [2]. Dietary manipulations during the developmentally plastic early postnatal period can lead to metabolic disorders [3] or improved health [4] later in life. Among other factors, the high prevalence of metabolic disorders is associated with an increase in fructose consumption [5]. Although fructose is mainly metabolised by the liver [6], it has been shown that other organs also play important roles in metabolizing a small amount of dietary fructose, including the small intestine, pancreas and testes [7]. Thus, dysregulation of fructose metabolism in primary tissues (particularly the liver), can alter fructose utilization in other tissues leading to the development of obesity, pancreatic and cardiovascular dysfunctions [7]. Excessive consumption of high-fructose diets can lead to increased epididymal fat deposition and reduction in testicular weight in males, and this can affect their reproductive performance [8]. High fructose diets can also cause haematological complications including altered red blood cell indices [e.g. packed cell volume (PCV)], haemoglobin (Hb) and mean corpuscular haemoglobin concentration (MCHC), thereby impacting on the physiological and pathological statuses of animals [9]. Additionally, high fructose diets can adversely affect cardiac mass thereby contributing to the development of cardiovascular complication [10].

The gastrointestinal tract (GIT) plays an important role in metabolism of dietary nutrients due to its digestive and absorptive capacity [11]. The development of the gut in rats is rapid during the suckling period thus dietary interventions during the neonatal period can cause alterations in GIT morphomtery and functional changes including precocious maturation of the GIT [12]. Precocious enteral fructose consumption in the early developmental stages upregulates the expression of GLUT-5 transporter (a highly specific fructose transporter) in the gut [13,14], thereby altering the morphometry of the GIT visceral and accessory organs that predisposes to the development of metabolic disorders later in adulthood.

Orally ingested phytochemicals during the early postnatal period can also affect the development of the gut and its accessory organs, thereby affecting the GIT morphometry and function, which in turn affects the growth of the growing animal [12]. Beneficial and adverse health outcomes were reported with the use of phytochemicals. For example, it was reported that orally ingested phytohaemagglutinin, a lectin from red kidney beans (*Phaseolus vulgaris*), causes hyperplastic growth of the intestines and pancreas in rats [15,16]. It also disrupts the brush borders of the inner

absorptive cells of the intestines, leading to diarrhoea and reduced body weight gain [17]. Additionally, ursolic acid [18] and oleanolic acid [19] were demonstrated to have a protective effects on the intestinal mucosal barrier and liver respectively.

While metabolic syndrome is well recognized as a consequence of high fructose diets, the impact of fructose on bone health is recognised [20]. Despite the physiological effects of sex steroids and growth hormone on long bones, it has been reported that sugar intake has a negative effect on bone mineralization in growing animals [20]. Srinaath et al. [21] have shown that zingerone possesses osteogenic properties in mice.

Having considered the impact of the neonatal zingerone administration on general metabolism (Chapter 3), non-alcoholic fatty liver disease (Chapter 4) and nephropathy (Chapter 5) in high-fructose diet fed rats, it was important to further establish the effect of the neonatal administration of zingerone on general health of the study rats. Thus, this chapter reports on the supplementary data which were collected on alternative measures of obesity, red blood cell indices, GIT and accessory visceral organ morphometry, masses of heart and pancreas and long bones' morphometry.

6.2 Materials and methods

6.2.1 Study setting, ethical approval, animal housing and study design

As described in the methods section of Chapters 3, 4 and 5.

6.2.2 Terminal procedures and measurements

The rats were euthanised as stated in Chapters 3, 4 and 5.

6.2.2.1 Obesity indices

After euthanasia, the terminal body length (nose-anus length) was measured using a measuring tape. The length (together with the terminal body mass) were used to determine the body mass index (BMI) and Lee obesity index which was computed as follows [22]:

BMI = body mass (g) / body length^2 (cm^2)

Lee index = cube root of body weight (g) / body length (cm)

6.2.2.2 Erythrocyte indices

A midline incision was then made ventrally on the thorax and abdomen, and intra-cardiac blood was drawn and used for determination of PCV and Hb concentration with a haematocrit-haemoglobin meter (InSight HCT Meter, Woodley Equipment Company Ltd, UK) as per the manufacturer's instructions. The PCV and Hb values were used to compute the mean corpuscular haemoglobin concentration (MCHC) as follows [23]:

MCHC (g/dL) = [Hb (g/dL) ÷ PCV (%)] × 100

Where MCHC = mean corpuscular haemoglobin concentration, Hb = haemoglobin, PCV = packed cell volume.

6.2.2.3 GIT morphometry and tissue masses

The heart, pancreas, stomach, small intestines, large intestines, caecum, testes and epididymal fat pads (males) were dissected out. The contents of the stomach, small and large intestines were carefully emptied. The masses of these tissues were determined using a Presica 310M balance (Precision Instruments, Johannesburg, South Africa). The lengths of small and large intestines were determined after they were carefully stretched on a dissection board and measured with a ruler. Empty carcasses following evisceration were also weighed (Snowrex Electronic Scale, Clover Scales, Johannesburg).

6.2.2.4. Long bones' morphometry

The long bones (femur and tibia) were dissected out, de-fleshed and oven-dried (Salvis®, Salvis Lab, Schweiz, Switzerland) at 40°C until constant mass for 5 days. The bones were then weighed with a balance (Snowrex Electronic Scale, Clover Scales, Johannesburg) to determine their dry mass, and their lengths measured with a pair of digital Vernier callipers [Major Tech (Pty) Ltd, KTV 150 digital calliper] to evaluate their growth.

Bone mass to length ratio (relative bone density) was then estimated as follows [24]:

Relative bone density = mass of bone (mg) / length of bone (mm)

6.2.3 Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) using GraphPad Prism 8 software (Graph-Pad Software Inc., San Diego, USA). The data were expressed as mean ± standard deviation. A multiple-comparisons Tukey *post hoc* test was used to compare the means, whilst a two-way ANOVA was used to compare the effect of sex, interaction and treatment variations. Statistical significance was considered at $P < 0.05$.

6.3 Results

6.3.1 Obesity indices

The BMI of male rats was not significantly different ($P = 0.6318$, ANOVA) across the different treatments. However, in female rats, the group that consumed only fructose had significantly ($P = 0.0036$, ANOVA) higher BMI compared to other treatment groups (Table 6.1). Neonatally administered zingerone prevented the fructose-induced increase in BMI in females, such that the group on combined fructose and zingerone had similar BMI ($P = 0.9997$, ANOVA) compared to the negative controls. Male rats had significantly higher BMI than their female counterparts [$P < 0.0001$ (sex effect), $P = 0.0448$ (treatment effect), $P = 0.4111$ (interaction effect), ANOVA].

There was no significant difference observed [Males ($P = 0.3151$, ANOVA); Females ($P = 0.4065$, ANOVA)] in the Lee index across the different treatment groups in both sexes (Table 6.1). There were no significant differences between the two sexes regarding the Lee index [$P = 0.3008$ (sex effect), $P = 0.2033$ (treatment effect), $P = 0.1919$ (interaction effect), ANOVA].

The absolute ($P = 0.4665$, ANOVA) and relative ($P = 0.2567$, ANOVA) masses of epididymal fat pads were not significantly different between all the treatment groups in male rats (Table 6.1).

Table 6.1: Effects of neonatally administered zingerone on the obesity indices of high-fructose fed rats in adulthood

Parameter	Sex	W	FS	ZF	ZW
BMI	Male	$0.64 \pm 0.05^{\#}$	$0.65 \pm 0.06^{\#}$	$0.63 \pm 0.04^{\#}$	$0.64 \pm 0.04^{\#}$
(g/cm^2)	Female	0.55 ± 0.02^{a}	0.60 ± 0.03^{b}	0.55 ± 0.05^{a}	0.55 ± 0.04^{a}
Lee index	Male	0.30 ± 0.01	0.30 ± 0.01	0.29 ± 0.01	0.30 ± 0.01
(g/cm)	Female	0.30 ± 0.01	0.31 ± 0.01	0.30 ± 0.01	0.30 ± 0.01
Epid. fat (g)	Male	3.00 ± 0.54	3.20 ± 0.62	3.30 ± 0.83	2.80 ± 0.65
Epid. fat (%BM)	Male	0.77 ± 0.11	0.83 ± 0.11	0.87 ± 0.14	0.76 ± 0.14

Data expressed as mean ± standard deviation. ^{ab} = within row means with different letters significantly different at P = 0.0036; $^{\#}$ = male rats had significantly higher BMI at P < 0.0001 compared to females; W = negative control (10 ml/kg of distilled water before weaning and unlimited access to plain tap water post weaning, n = 20; 9 males, 11 females); FS = 10 ml/kg of fructose solution at 20% before weaning and unlimited access to fructose solution post weaning, n = 20; 9 males, 11 females); ZF = 10 ml/kg of fructose solution at 20% plus 40 mg/kg of zingerone dissolved in 10 ml/kg of distilled water before weaning and unlimited access to plain tap water post weaning, n = 21; 9 males, 12 females); ZW = 40 mg/kg of zingerone dissolved in 10 ml/kg of distilled water before weaning and unlimited access to plain tap water post weaning, n =18; 8 males, 10 females); %BM = relative mass of organ expressed as a percentage of body mass; BMI = body mass index; Epid = epididymal.

6.3.2 Erythrocyte indices

In males, rats that received zingerone with or without fructose had significantly higher PCV (P = 0.0002, ANOVA) and Hb content (P = 0.0633, ANOVA) compared to negative controls (Table 6.2). The MCHC was not significantly (P = 0.6794, ANOVA) different across the different treatment groups.

The PCV (P = 0.6074; ANOVA), MCHC (P = 0.0571, ANOVA) and concentrations of Hb (P = 0.5877, ANOVA) were not significantly different across all the treatment groups in female rats.

Male rats had significantly higher concentrations of Hb [P = 0.0424 (sex effect), P = 0.2419 (treatment and interaction effects), ANOVA] than female rats (Table 6.2). However, there were no significant differences between the two sexes regarding the PCV [P = 0.3181 (sex effect), P = 0.1128 (treatment effect), P = 0.5549 (interaction effect), ANOVA] and MCHC [P = 0.2543 (sex effect), P = 0.0618 (treatment effect), P = 0.8099 (interaction effect), ANOVA].

Table 6.2: Effects of neonatally administered zingerone on clinical haematological parameters of high-fructose fed rats in adulthood

Parameter	Sex	W	FS	ZF	ZW
PCV (%)	Male	53.00 ± 3.20^{a}	55.00 ± 2.20^{ab}	56.00 ± 4.00^{b}	58.00 ± 3.00^{b}
	Female	54.00 ± 2.60	54.00 ± 4.20	55.00 ± 4.00	56.00 ± 2.00
Hb (g/dL)	Male	18.00 ± 1.00$^{a\#}$	18.00 ± 0.69$^{ab\#}$	19.00 ± 1.30$^{b\#}$	19.00 ± 0.95$^{b\#}$
	Female	18.00 ± 0.85	18.00 ± 1.40	18.00 ± 1.30	18.00 ± 0.64
MCHC (g/dL)	Male	33.32 ± 0.19	33.31 ± 0.18	33.37 ± 0.15	33.26 ± 0.15
	Female	33.23 ± 0.12	33.27 ± 0.16	33.38 ± 0.18	33.21 ± 0.16

Data expressed as mean ± standard deviation. ^{ab} = within row means with different letters in superscript significantly different at P = 0.0002 (PCV) and P = 0.0633 (Hb); $^{\#}$ = male rats had significantly higher Hb concentrations than female rats at P = 0.0424; W = negative control (10 ml/kg of distilled water before weaning and unlimited access to plain tap water post weaning, n = 20; 9 males, 11 females); FS = 10 ml/kg of fructose solution at 20% before weaning and unlimited access to fructose solution post weaning, n = 20; 9 males, 11 females); ZF = 10 ml/kg of fructose solution at 20% plus 40 mg/kg of zingerone dissolved in 10 ml/kg of distilled water before weaning and unlimited access to plain tap water post weaning, n = 21; 9 males, 12 females); ZW = 40 mg/kg of zingerone dissolved in 10 ml/kg of distilled water before weaning and unlimited access to plain tap water post weaning, n = 18; 8 males, 10 females); PCV = packed cell volume; Hb = haemoglobin; MCHC = mean corpuscular haemoglobin concentration.

6.3.3 Visceral organ gross morphometry

The absolute and relative masses of stomach, intestines and accessory organs of the GIT, and the absolute and relative lengths of small and large intestines were not significantly ($P > 0.05$, ANOVA) different across the different treatment groups in both sexes (Table 6.3).

The absolute ($P = 0.6904$, ANOVA) and relative ($P = 0.9907$, ANOVA) masses of testes were not significantly different between all the treatment groups in male rats.

The absolute masses of the organs were significantly ($P < 0.0001$, ANOVA) higher in males than females, whereas the masses relative to body weight were significantly ($P < 0.0001$, ANOVA) higher in females than male rats (Table 6.3). The absolute lengths of small and large intestines were significantly ($P < 0.0001$, ANOVA) higher in males than females, whereas the relative lengths of small and large intestines were significantly ($P < 0.0001$, ANOVA) higher in females than male rats.

Table 6.3: Effects of neonatally administered zingerone on the morphometry of GIT visceral organs, masses of the heart, pancreas and testes of high-fructose fed rats

Parameter	Sex	W	FS	ZF	ZW
Heart (g)	Male	1.30 ± 0.16$^{\#}$	1.20 ± 0.17$^{\#}$	1.20 ± 0.13$^{\#}$	1.20 ± 0.09$^{\#}$
	Female	0.86 ± 0.066	0.93 ± 0.05	0.93 ± 0.13	0.87 ± 0.07
Heart (%BM)	Male	0.33 ± 0.02	0.32 ± 0.02	0.32 ± 0.01	0.33 ± 0.02
	Female	0.36 ± 0.03$^{\lambda}$	0.35 ± 0.02$^{\lambda}$	0.38 ± 0.07$^{\lambda}$	0.37 ± 0.03$^{\lambda}$
Pancreas (g)	Male	1.30 ± 0.25$^{\#}$	1.40 ± 0.20$^{\#}$	1.30 ± 0.12$^{\#}$	1.20 ± 0.34$^{\#}$
	Female	1.00 ± 0.19	1.20 ± 0.12	1.00 ± 0.16	1.10 ± 0.08
Pancreas	Male	0.34 ± 0.06	0.36 ± 0.06	0.35 ± 0.04	0.33 ± 0.08
(%BM)	Female	0.42 ± 0.06$^{\lambda}$	0.44 ± 0.05$^{\lambda}$	0.42 ± 0.06$^{\lambda}$	0.45 ± 0.04$^{\lambda}$
Stomach (g)	Male	1.70 ± 0.18$^{\#}$	1.80 ± 0.22$^{\#}$	1.90 ± 0.26$^{\#}$	1.70 ± 0.14$^{\#}$
	Female	1.30 ± 0.08	1.50 ± 0.17	1.40 ± 0.14	1.40 ± 0.13
Stomach	Male	0.48 ± 0.06	0.47 ± 0.03	0.48 ± 0.04	0.48 ± 0.04
(%BM)	Female	0.54 ± 0.03$^{\lambda}$	0.55 ± 0.06$^{\lambda}$	0.57 ± 0.05$^{\lambda}$	0.58 ± 0.04$^{\lambda}$
Caecum (g)	Male	1.40 ± 0.25$^{\#}$	1.20 ± 0.22$^{\#}$	1.20 ± 0.22$^{\#}$	1.30 ± 0.13$^{\#}$
	Female	0.99 ± 0.18	0.91 ± 0.15	0.89 ± 0.18	0.98 ± 0.11
Caecum	Male	0.37 ± 0.07	0.31 ± 0.04	0.32 ± 0.04	0.35 ± 0.03
(%BM)	Female	0.44 ± 0.08$^{\lambda}$	0.39 ± 0.07$^{\lambda}$	0.39 ± 0.08$^{\lambda}$	0.42 ± 0.06$^{\lambda}$
S. Intestine (g)	Male	9.20 ± 0.76$^{\#}$	9.40 ± 0.99$^{\#}$	9.10 ± 0.95$^{\#}$	8.40 ± 0.74$^{\#}$
	Female	6.50 ± 0.38	6.80 ± 0.83	6.70 ± 0.54	6.70 ± 0.62
S. intestine	Male	2.40 ± 0.25	2.40 ± 0.14	2.40 ± 0.17	2.30 ± 0.17
(%BM)	Female	2.70 ± 0.13$^{\lambda}$	2.70 ± 0.19$^{\lambda}$	2.80 ± 0.20$^{\lambda}$	2.70 ± 0.19$^{\lambda}$
L. intestine (g)	Male	1.70 ± 0.35$^{\#}$	1.70 ± 0.20$^{\#}$	1.60 ± 0.16$^{\#}$	1.80 ± 0.16$^{\#}$
	Female	1.50 ± 0.30	1.40 ± 0.18	1.30 ± 0.20	1.30 ± 0.31
L. intestine	Male	0.45 ± 0.11	0.45 ± 0.04	0.42 ± 0.03	0.49 ± 0.04
(%BM)	Female	0.61 ± 0.13$^{\lambda}$	0.52 ± 0.07$^{\lambda}$	0.54 ± 0.09$^{\lambda}$	0.53 ± 0.12$^{\lambda}$
S. intestine	Male	1330.00 ± 89.00$^{\#}$	1371.00 ± 157.00$^{\#}$	1334.00 ± 51.00$^{\#}$	1334.00 ± 84.00$^{\#}$
length (mm)	Female	1188.00 ± 41.00	1228.00 ± 64.00	1178.00 ± 40.00	1187.00 ± 50.00
	Male	234.00 ± 15.00$^{\#}$	223.00 ± 18.00$^{\#}$	224.00 ± 8.80$^{\#}$	231.00 ± 15.00$^{\#}$

L. intestine length (mm)	Female	209.00 ± 10.00	207.00 ± 9.80	205.00 ± 8.30	209.00 ± 8.50
Testes (g)	Male	5.10 ± 0.39	5.20 ± 0.48	4.90 ± 0.36	4.90 ± 0.74
Testes (%BM)	Male	1.30 ± 0.09	1.30 ± 0.07	1.30 ± 0.09	1.30 ± 0.25

Data expressed as mean ± standard deviation. # = male rats had significantly higher absolute organ masses and lengths than female rats (P < 0.05); λ = female rats had significantly higher relative organ masses than male rats (P < 0.05); %BM = relative mass of organ expressed as a percentage of body mass; W = negative control (10 ml/kg of distilled water before weaning and unlimited access to plain tap water post weaning, n = 20; 9 males, 11 females); FS = 10 ml/kg of fructose solution at 20% before weaning and unlimited access to fructose solution post weaning, n = 20; 9 males, 11 females); ZF = 10 ml/kg of fructose solution at 20% plus 40 mg/kg of zingerone dissolved in 10 ml/kg of distilled water before weaning and unlimited access to plain tap water post weaning, n = 21; 9 males, 12 females); ZW = 40 mg/kg of zingerone dissolved in 10 ml/kg of distilled water before weaning and unlimited access to plain tap water post weaning, n = 18; 8 males, 10 females); S = small; L = large.

6.3.4 Empty carcass mass and long bones parameters

Table 6.4 shows the effects of neonatally administered zingerone on empt**y** carcass mass and morphometry of long bones of high-fructose fed rats in adulthood. There were no significant differences on the empty carcass mass ($P = 0.4795$, ANOVA) and long bones parameters ($P > 0.05$, ANOVA) across the different treatment groups in male rats.

In female rats, the group that was administered fructose only had significantly higher empty carcass mass ($P = 0.0025$, ANOVA), femoral mass ($P = 0.0076$, ANOVA), and femoral length ($P = 0.0192$, ANOVA), compared to the remaining groups. Rats on fructose only had significantly higher tibial mass ($P = 0.0131$, ANOVA), and tibial densities ($P = 0.0217$, ANOVA), compared to those on zingerone only. There were no significant differences in the femoral densities and tibial length ($P > 0.05$, ANOVA).

Male rats had significantly higher empty carcass mass and long bones parameters than the corresponding female rats (Sex effect: $P < 0.0001$, ANOVA).

Table 6.4: Effects of neonatally administered zingerone on empty carcass mass and long bones of high-fructose fed rats in adulthood

Parameter	Sex	W	FS	ZF	ZW
Empty carcass mass (g)	Male	297.00 ± 27.00$^{\#}$	307.00 ± 38.00$^{\#}$	287.00 ± 30.00$^{\#}$	287.00 ± 24.00$^{\#}$
	Female	186.00 ± 10.00^{a}	201.00 ± 7.10^{b}	185.00 ± 12.00^{a}	182.00 ± 15.00^{a}
Femur mass (mg)	Male	595.00 ± 42.00$^{\#}$	598.00 ± 60.00$^{\#}$	590.00 ± 42.00$^{\#}$	583.00 ± 35.00$^{\#}$
	Female	471.00 ± 37.00^{a}	514.00 ± 24.00^{b}	482.00 ± 33.00^{a}	467.00 ± 37.00^{a}
Femur length (mm)	Male	35.00 ± 0.94$^{\#}$	35.00 ± 1.90$^{\#}$	34.00 ± 1.10$^{\#}$	34.00 ± 0.54$^{\#}$
	Female	32.00 ± 1.30^{a}	33.00 ± 0.59^{b}	32.00 ± 0.70^{a}	32.00 ± 0.86^{a}
Femur R.D. (mg/mm)	Male	17.00 ± 1.10$^{\#}$	17.00 ± 1.20$^{\#}$	17.00 ± 0.90$^{\#}$	17.00 ± 1.10$^{\#}$
	Female	15.00 ± 0.76	16.00 ± 0.67	15.00 ± 1.00	15.00 ± 0.87
Tibia mass (mg)	Male	397.00 ± 37.00$^{\#}$	400.00 ± 39.00$^{\#}$	396.00 ± 36.00$^{\#}$	384.00 ± 27.00$^{\#}$
	Female	306.00 ± 17.00^{ab}	327.00 ± 13.00^{b}	305.00 ± 25.00^{ab}	297.00 ± 27.00^{a}
Tibia length (mm)	Male	38.00 ± 0.62$^{\#}$	37.00 ± 1.50$^{\#}$	38.00 ± 1.20$^{\#}$	38.00 ± 0.80$^{\#}$
	Female	35.00 ± 0.77	35.00 ± 0.35	35.00 ± 0.64	35.00 ± 0.91
Tibia R.D. (mg/mm)	Male	10.00 ± 0.86$^{\#}$	11.00 ± 0.68$^{\#}$	10.00 ± 0.74$^{\#}$	10.00 ± 0.61$^{\#}$
	Female	8.80 ± 0.41^{ab}	9.30 ± 0.33^{b}	8.80 ± 0.66^{ab}	8.60 ± 0.61^{a}

Data expressed as mean ± standard deviation. ^{ab} = within row means with different letters in superscript significantly different at $P < 0.05$; $^{\#}$ = male rats had significantly higher empty carcass mass and long bones parameters than female rats at $P < 0.0001$; W = negative control (10 ml/kg of distilled water before weaning and unlimited access to plain tap water post weaning, n = 20; 9 males, 11 females); FS = 10 ml/kg of fructose solution at 20% before weaning and unlimited access to fructose solution post weaning, n = 20; 9 males, 11 females); ZF = 10 ml/kg of fructose solution at 20% plus 40 mg/kg of zingerone dissolved in 10 ml/kg of distilled water before weaning and unlimited access to plain tap water post weaning, n = 21; 9 males, 12 females); ZW = 40 mg/kg of zingerone dissolved in 10 ml/kg of distilled water before weaning and unlimited access to plain tap water post weaning, n = 18; 8 males, 10 females); R.D.= relative density.

6.4 Discussion

The current study was conducted to investigate the potential long-term protective effect of neonatally administered zingerone on the general health status and extra growth parameters secondary to high-fructose diets in male and female rats. We showed that neonatal administration of zingerone orally to high-fructose fed rats had no long-term adverse effects on general health status and GIT morphometry of the rats in both sexes. However, BMI and growth parameters were negatively affected by the high-fructose diet in adult female rats, which was prevented by the neonatal zingerone administration. Sexually dimorphic outcomes were observed in that male rats had higher BMI, Hb concentrations, absolute organ masses and long bones' morphometry than their female counterparts.

Lee obesity index and BMI are positively correlated with body fat percentage in rats and are thus used as an indirect indicators of body fat percentage [25,26]. A Lee index greater than 0.3 is an indication of obesity in rats [25]. In the current study, the interventions did not affect the Lee obesity index of the study rats in both sexes, implying that the rats did not develop long-term overt obesity based on this index. Araujo et al. [27] also reported similar finding on obesity index following consumption of 10% fructose solution to adolescent male Wistar rats for 8 weeks, the finding they attributed to the period of fructose consumption which is was not long enough to induce changes to the index.

However, we observed an increase in BMI following consumption of excess fructose solution by the female rats, a finding that was not observed in male rats. The increase in BMI of female rats is in agreement with the finding we reported in Chapter 3, in which the female rats had developed long-term increased terminal body mass and visceral adiposity following consumption of 20% fructose solution, which are important indicators for the development of obesity [28]. Novelli et al. [22] reported similar finding on BMI and Lee index following consumption of 30% sucrose solution by the adolescent male Wistar rats for 30 days. This finding indicates that there might be a discrepancy in the sensitivity of the indices, thus future studies would require further sensitivity analysis for obesity markers.

Despite the male rats on fructose alone developing visceral adiposity as we have reported in Chapter 3, the rats had similar BMI across the different treatment groups. This further reinforces our finding in Chapter 3 where the male rats had similar terminal body mass across different treatments. Thus, it could be speculated that despite the increased visceral adiposity observed on

dissection and weighing on the visceral fat, its accretion had not reached the threshold level for inducing significant changes to the BMI and Lee index [25] in the males. Thus, reinforcing the need for use of methods to quantify the visceral fat quantitatively rather than the proxy measures. Since BMI does not differentiate between fat and lean masses [29], and the fact that female rats had higher visceral fat percentage than males (Chapter 3), the higher BMI of male rats could be attributed to the increased muscle mass associated with male rats [30]. This effect has been ascribed to increased activities of testosterone and growth hormone in male rats [31,32], this could be reflected by the higher terminal body masses of male rats compared to females reported in Chapter 3.

Epididymal fat is a subset of the general abdominal adiposity [33]. Increased epididymal fat is associated with oxidative and inflammatory processes that lead to epididymitis and sexual dysfunction in males [34]. The interventions of the current study did not affect the epididymal fat mass of the males. This had reinforced our findings on body mass, BMI and Lee index of male rats. Fakhoury-Sayegh et al. [35] reported similar observation on the epididymal fat when 20% fructose enriched-diet was consumed by adolescent male Wistar rats for 16 weeks. However, Crescenzo et al. [36] reported a contradictory finding as epididymal fat was increased in adult male Sprague Dawley rats that consumed fructose-rich diets for eight weeks. The age of the rats could be responsible for the contrasting findings since adult rats are more prone to fructose-induced metabolic derangements [37].

It would be observe that the visceral adiposity (which is wrongly considered as general intra-abdominal adiposity) of male rats was increased as reported in Chapter 3. Despite epididymal fat is considered part of the general abdominal fat, however, not all intra-abdominal fats surround the visceral organs, and this includes epididymal fat since testis/epididymis are not visceral organs [33]. Thus, increased visceral fat could not always translate to increased epididymal fat, hence correlates well with the finding of this study.

Red blood cell parameters particularly PCV, Hb and MCHC are important tools used for assessing the health and pathological statuses of animals [38]. They reflect alterations that may occur during the course of a disease, as well as evaluate the efficacy of management interventions [9]. Whilst PCV (haematocrit) is the percentage of red cells in the blood and Hb is a protein contained within the red cells which is responsible for oxygen transport, MCHC is the ratio of Hb weight to the volume of an average red cell in a population of cells [23]. These red cell

indices can be affected by deficiency of iron, vitamin B12 or folic acid [39]. For instance, iron deficiency results in microcytic-hypochromic anaemia, whilst vitamin B_{12} and folic acid deficiencies result in megaloblastic anaemia [39]. In addition, the bioavailability of these nutrients can be affected by phytochemicals; it has been reported that phytates can decrease the absorption and bioavailability of iron in the GIT [40]. It is therefore necessary to evaluate the red cell status of the study rats by determining the PCV, Hb and MCHC [9].

The current study observed an increase in PCV and Hb concentration in the zingerone treated groups compared to the controls in male rats, with no significant changes in the red cell indices of female rats. This implies that zingerone could be a haematinic phytochemical that could be safe for administration without adversely affecting the red blood cells indices in both sexes. The haematinic-normochromic effect of zingerone observed in this study could be attributed to its ability to promote erythropoietin secretion, a hormone that stimulates formation of red blood cells in the bone marrow [41]. Additionally, all the mean values of Hb, PCV and MCHC recorded in the current study were slightly higher than the normal ranges previously reported for rats in the age set and sex [41–43]. The higher values could be attributed to the relatively moderate altitude of Johannesburg (1, 753 meters above sea level) where the study was conducted [44]. It has been reported that at moderate to high altitudes, there is a compensatory mechanism in response to tissue hypoxia, causing the release of erythropoietin that leads to increased erythropoiesis, which translates to increased red cell indices [45].

The higher red blood cells' indices observed with male rats in this study could be due to the testosterone effect of activating red cell production via stimulation of erythropoietin release [42]. In addition to impact on red cell indices, GIT and accessory organs can be affected by dietary interventions.

Oral administration of plants' phytochemicals may affect the digestion and absorption of dietary nutrients in the gut, and this can affect the development of the gut and its accessory organs thereby interfering with the animal's growth [46]. For example, flavonoids and phytates can chelate iron and zinc thereby reducing the absorption of these micronutrients; they can also reduce the activities of digestive enzymes by forming complexes with dietary carbohydrates or proteins [46]. Additionally, ingestion of high-fructose diets can cause cardiovascular and pancreatic complications that affect their individual organ masses. For example, excess dietary fructose is metabolized and converted into fat mainly by the liver, and deposited at the ectopic sites

including pancreas and blood vessels [47]. In the pancreas, the excess fat may affect the beta cells of the pancreas impairing insulin secretion, and can lead to pancreatic damage [47]. The excess fat may also be deposited in blood vessels inform of cholesterol and low-density lipoprotein, which leads to narrowing of the arteriolar walls, resistance to blood flow, hypertension and eventually cardiac failure [47]. However, the interventions of the current study did not adversely affect the masses of the GIT organs, heart, pancreas (both sexes) and testes (males) in adult life. Fakhoury-Sayegh et al. [35] also did not observed changes in the weights of pancreas when 20% fructose enriched-diet was consumed by adolescent male Wistar rats for 16 weeks. The absence of changes in the abdominal and thoracic organs implies that zingerone did not negatively affect the heart, pancreas and absorption of dietary nutrients in the gut of the rats in this study.

The significantly greater absolute weights of the GIT organs, heart and pancreas compared to their matched females was probably due to the effects of testosterone which promotes tissue growth, the concentrations of which are usually higher in adult males compared to females [32]. The fact that female rats had greater relative organ weights than males could be due to inherent body sizes of the male rats when compared with females, since male rats have greater muscle mass and organ weight than female rats [30,32,48].

Empty carcass mass is a good indicator of growth performance since the effect of GIT and bladder residual content is avoided [49]. Although administration of 20% fructose solution did not affect the empty carcass masses of the male rats, the empty carcass masses of the fructose-fed group was heavier than the control group in female rats. This is not surprising since female rats are more susceptible to fructose-induced metabolic derangements that their male counterparts [50]. The fructose-induced increase in empty carcass mass in female rats was unlikely due to lean body mass since male rat have greater muscle mass than females [30]. In Chapter 3, we have reported increase in terminal body mass (females only) and visceral fat accumulation (both sexes) secondary to excess fructose consumption. Thus, we speculate that the heavier carcass mass of female rats (which was not seen with males) could be due to subcutaneous fat accumulation since fructose has been reported to contribute to subcutaneous fat synthesis [36]. The fructose-induced increase in empty carcass mass observed in females was prevented by neonatal administration of zingerone, implying that zingerone had provided long-term protection against

fructose-induced increase in carcass mass of female rats. This beneficial effect of zingerone could be attributed to its ability to regulate bone metabolism in rodents [21].

Male rats had heavier empty carcass masses compared to their corresponding females; this is not surprising since male Sprague Dawley rats are naturally bigger than females due to the greater muscle mass associated with male rats compared to females [51]. This could be due to the greater effect (secondary to higher concentrations) of testosterone in males, a hormone responsible for promoting tissue growth [32].

Long-term growth performance can further be assessed using long bones' indices because the length of the bones does not usually change acutely during the growing phase, despite the effect of growth hormone on long bones [52]. High- fructose diet has been shown to lower the levels of osteocalcin (a marker of bone formation) thereby affecting the growth of bones [53]. In the current study, administration of 20% fructose solution did not affect the long bones morphometry of the male rats. However, the mass (femur and tibia), length (femur) and density (tibia) of the fructose-fed group were increased in female rats. This is could be attributed to the increased BMI observed with female rats in this study, since fructose-induced increased BMI (which is a measure of obesity) has been shown to affect the morphometry of long bones [54]. This was reversed by the neonatal administration of zingerone, implying that zingerone had provided long-term protection against fructose-induced morphometric changes on long bones of female rats. The long bones indices of the male rats that was greater compared to females in matched groups could probably be attributed to the increased activity of growth hormone secondary to high testosterone levels [32].

6.5 Conclusion

The findings indicated that consumption of 20% fructose solution for 12 weeks did not adversely affect the haematological proxies of general health, GIT gross morphometry and accessory organs of both male and female rats. However, the fructose solution had increased the BMI and long bones' morphometry of the female rats, which were prevented by the neonatal administration of zingerone. This implies that while administration of zingerone to neonates does not have long-term deleterious effect to the overall health status of the animals, future studies need to critically examine the mechanisms by which zingerone exerts its protective effects on the long bones in high fructose fed individuals.

The following chapter presents an overall discussion of Chapters 3, 4, 5 and 6, in which all the major findings of the individual chapters are concisely articulated and contextualised.

6.6 References

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CHAPTER SEVEN - General discussion

7.1 Introduction

This study evaluated whether orally administered zingerone in the neonatal period would protect against the long-term development of dietary fructose-induced metabolic dysfunction, fatty liver, nephropathy and alteration in the general health status in male and female Sprague Dawley rats. It was observed in adulthood that rats provided with fructose solution consumed more fluid, ate less food (standard rat chow) and had a greater total calorie intake than those that had plain drinking water in both sexes. Additionally, rats that consumed only fructose had increased terminal body masses (females only), visceral adiposity, elevated plasma TGs and cholesterol concentrations in adulthood in both sexes (metabolic dysfunction).

Rats that consumed only fructose had long-term increased hepatic lipid accretion and steatosis in both sexes (fatty liver). Furthermore, rats that consumed fructose only had increased plasma KIM-1 and decreased urinary space at termination in both sexes (early features of nephropathy). The empty carcass mass and long bones' densities were increased by high-fructose diet in female rats only. All these fructose-induced derangements and pathologies were prevented by neonatally administered zingerone, implying that zingerone had programmed protection against high-fructose diet-induced metabolic derangements. Otherwise, no long-term adverse effects on general health status were observed following neonatal administration of oral zingrone to fructose-fed rats in both sexes. However, sexually dimorphic responses indicated that female rats are more susceptible to fructose-induced metabolic derangements than male rats.

Central obesity, dyslipidaemia, hyperglycaemia and NAFLD are amongst the major components of metabolic syndrome [1]. These metabolic abnormalities can affect the renal and general health physiology if not promptly addressed [2–4]. The current study observed that following a long-term consumption of high-fructose solution, the study rats had developed adiposity, dyslipidaemia and fatty liver, which were complicated by renal involvement. Thus, excluding hyperglycaemia, the rats had developed a nearly full-blown metabolic syndrome in adulthood, and neonatal administration of oral zingerone had programmed for long-term protection against the syndrome. Visceral obesity and its associated dyslipidaemia are the early features of fructose-induced metabolic dysfunction observed in the current study.

7.2 Development of metabolic dysfunction

Although waist circumference is considered a major determinant of central obesity [5], however the current study quantified the actual visceral fat pad (visceral adiposity) since it is the major determinant of central (abdominal) obesity [6]. In addition, BMI was also evaluated in this study. Despite the fact that BMI does not differentiate between muscle and fat mass, however it (BMI) correlates well with various cardio-metabolic risk factors including dyslipidaemia [7]. This is evident in this study as high-fructose solution had programmed for long-term development of increased adiposity (both sexes; Chapter 3), BMI (females only; Chapter 6) hepatic lipid accumulation (both sexes; Chapter 4) and dyslipidaemia (both sexes; Chapter 3) in the study rats. These metabolic derangements are strongly associated with cardiovascular diseases [5]. Interestingly, neonatally administered zingerone had programmed for long-term protection against the fructose-induced metabolic dysfunctions as reported in Chapter 3. However, despite the merits and demerits of BMI over waist circumference being debated, it would be best to assess the risk and progression of metabolic syndrome by measuring both the BMI and waist circumference [8], as both predict the risk of developing insulin resistance, hyperglycaemia, and type 2 diabetes [5].

Both male and female rats did not develop insulin resistance or hyperglycaemia in this study, despite the fact rats had developed visceral obesity (Chapter 3). This is not surprising since it has been shown that visceral obesity precedes the development of type 2 diabetes [1]. The fact that the rats did not develop hyperglycaemia had been reinforced by the findings on insulin, HOMA-IR, adiponectin (Chapter 3) and pancreas (Chapter 6), all of which were not affected by the interventions in this study. As stated in Chapter 2, insulin resistance and hyperglycaemia could be due to oxidative and inflammatory processes associated with metabolic dysfunction [9]. It would be observed that the levels of TBARS, IL-6 and TNF-α were not affected by the interventions of this study (Chapter 4), and this is a reflection of normoglycaemia observed in chapter 3, on the levels of fasting blood glucose. Although oral glucose tolerance test (OGTT) can be used to diagnose type 2 diabetes, however the current study opted for fasting blood glucose because OGTT is heavy, difficult, poorly tolerated and consumes so much time [10]. Additionally, six-hour fasting blood glucose correlates well with chronic hyperglycaemia than OGTT [11].

The fact that female rats were more prone to fructose-induced metabolic dysfunction in this study has been discussed in chapter 3. In addition to visceral obesity [1], type 2 diabetes is also a risk factor for the development of NAFLD [8,12].

7.3 Development of non-alcoholic fatty liver disease

As stated in chapter 4, NAFLD is the hepatic manifestation of metabolic syndrome as it (NAFLD) is invariably associated with the components of metabolic syndrome, including visceral obesity, dyslipidaemia, insulin resistance, elevated blood glucose levels and diabetes [1,13]. There are two major and progressive causes of liver injury that can lead to the development of NAFLD and NASH, and these include hepatic lipid accretion and oxidative stress [12]. The progressive nature of NAFLD involves the "two-hit" theory: the first hit being the hepatic lipid deposition and the second hit being the oxidative stress, which stimulates hepatocytes' injury resulting to development of NASH [12]. This "two hit" theory is partially established in this study as evident by hepatic steatosis (Chapter 4) as the first hit in both male and female rats; and the liver is yet to be affected by the second hit as evident by the insignificant levels of oxidative and inflammatory markers, TBARS, MDA, IL-6 and TNF-α (Chapters 3, 4 and 5). This finding also correlates well with the glycaemic parameters that were not significantly affected by the interventions in both sexes (Chapter 3), implying that there was no significant oxidative damage that could alter insulin sensitivity or glucose metabolism.

Despite the issue of NAFLD as a cause or a result of insulin resistance or glucose intolerance [13], it has been reported that NAFLD precedes the development of type 2 diabetes and full blown metabolic syndrome, even in patients with normal BMI [1], since 56% of patients with NAFLD are non-diabetics [13]. This is similar to the finding of the current study where male rats were observed to develop hepatic steatosis (Chapter 4) without affecting their BMI (Chapter 6). The fact that male rats developed fatty liver without changes in their BMIs is not surprising since hepatic lipid content is independently associated with the components of metabolic syndrome [1].
The long-term fructose-induced hepatic steatosis was prevented by neonatal administration of oral zingerone in both sexes as described in Chapter 4.

Although it has been claimed that males have a higher risk for developing hepatic steatosis than females [1], current study did not observe dimorphic response to development of fructose-induced

fatty liver. Extrahepatic manifestations of NAFLD include renal involvement and can lead to chronic kidney disease [8].

7.4 Development of nephropathy

In this study, long-term high-fructose consumption had led to the development of early features of nephropathy via elevated plasma KIM-1 levels and decreased urinary space in both sexes (Chapter 5). These features are invariably associated with diabetic nephropathy [14]. Although the levels of other markers of renal damage including uric acid, oxidative and inflammatory markers (Chapters 4 and 5) were not significantly altered in this study, we could conclude that fructose consumption had induced renal injury in the rats since KIM-1 is not expressed in normal renal parenchyma but expressed only in injured renal cells [15].

Although diabetes is the major cause of diabetic nephropathy globally, it has been shown that obesity can lead to poor renal outcomes independent of diabetes [11,16]. This is in agreement with finding of this study in which the glycaemic parameters were not affected by the interventions (Chapter 3) despite development of early features of nephropathy by both male and female rats (Chapter 5). This implies that administration of fructose solution had programmed for long-term development of early features of nephropathy in rats, without inducing diabetes.

Neonatally administered zingerone had programmed for long-term protection against the development of early features of nephropathy (Chapter 5). Similar protective effects of zingerone against diabetic nephropathy were demonstrated by Rehman et al. [17] and Cui et al. [16] in rats and mice respectively. It has been shown that nephropathies can affect the general health status including red blood cell indices in patients with metabolic syndrome [18].

7.5 Alterations to the general health markers and extra growth parameters

Routinely, red blood cell indices are used in various blood tests for quantification and physical characterization of erythrocytes [18]. Altered red cell indices can lead to insulin resistance secondary to reduction in the blood flow, thereby decreasing flow of oxygen, glucose and insulin to the tissues including the red blood cells [18]. This renders the erythrocytes to cellular oxidative stress and inflammatory processes that may be associated with components of metabolic syndrome [18].

Except for zingerone treated groups that had higher PCV and Hb concentration in male rats, the interventions of the current study did not affect the red blood cell indices in the study rats

(Chapter 6). This finding could be reinforced by the levels of oxidative stress markers (MDA and TBARS) and inflammatory cytokines (IL-6 and TNF-α) that were not affected by the current study interventions (Chapters 4 and 5). Thus, this indicated that zingerone could be safe for administration in the neonatal period to provide long-term protection against fructose-induced metabolic derangements. This finding also showed that zingerone could be used as a haematinic agent to manage anaemic conditions when ingested orally via the GIT.

In the first few weeks of life, the immature GIT of rats undergoes rapid functional and structural developmental changes [19], such that dietary interventions during this plastic period can positively or adversely affect the structure and or function of the GIT later in life [20]. As such, evaluation of GIT morphometry could explore the impact of dietary interventions on the GIT physiology.
The interventions of the current study did not affect the morphometry of the GIT, implying that neonatal administration of zingerone did not adversely affect the GIT structure, since it has been reported that phytochemicals (including lectin from red kidney bean) can cause precocious maturation of GIT in suckling rats [19]. However, a previous study had reported similar finding in which the GIT morphometry was not affected following administration of dietary interventions in neonatal rats [21]. The finding of this study on GIT could be reinforced by that of chapter 4 and 5, in which the oxidant and inflammatory markers (which are also implicated in the pathogenesis of GIT dysfunction [22]) were not affected by the interventions. GIT function influences growth parameters in growing animals [23].
The current study observed greater empty carcass mass and long bones' densities in female rats that consumed only fructose. This has contrasted the findings of previous studies in which excess fructose consumption did not affect the empty carcass mass and morphometry of long bones in growing rats of both sexes [21,24,25]. This is not surprising since there have been conflicting reports on the effect of fructose on bone health, in which either beneficial or adverse effects of excess fructose consumption on bones were observed [26,27]. The effect of fructose on empty carcass mass and long bones of female rats observed in this study could be reinforced by the increased terminal body mass (Chapter 3) and BMI (Chapter 6) observed with the females in the same treatment group. However, neonatal zingerone administration had conferred a long-term protection against fructose-induced changes on extra growth parameters (Chapter 6).

The next chapter summarises the findings of this study, highlights some limitations and provides recommendations for future studies.

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CHAPTER EIGHT - Summary, limitations and future directions

8.1 Summary and conclusion

The current study evaluated the potential therapeutic effects of orally administered zingerone to neonatal male and female Sprague Dawley rats on the development of metabolic derangements induced by long-term consumption of a fructose-rich diet, specifically on four focus areas:

1. Metabolic dysfunction [By measuring/evaluating fluid and feed intake, total calorie intake, body mass and length, BMI, Lee index, FBG, TGs, cholesterol subtypes, insulin, adiponectin, HOMA-IR index, visceral fat and epididymal fat pad (males)].
2 Non-alcoholic fatty liver disease (By measuring/evaluating ALT activity, liver mass, hepatic lipid content, TBARS, TNF-α, IL-6, and liver histology).
3. Nephropathy (By measuring/evaluating kidney mass, uric acid, KIM-1, MDA and kidney histomorphometry).
4. General health [By measuring/evaluating erythrocyte PCV, Hb, MCHC, morphometry of stomach, small intestines, large intestines and caecum; masses of heart, pancreas and testes (males); empty carcass mass and linear growth].

This study has demonstrated that long-term fructose consumption had programmed for the development of visceral obesity and dyslipidaemia (features of metabolic dysfunction), hepatic lipid accretion and steatosis (features of NAFLD), elevated KIM-1 levels and decreased urinary space area (early features of nephropathy), in both male and female rats. The ability of fructose to induce long-term metabolic derangements have been attributed to its interaction with the early developmental period of plasticity (including early postnatal period), dysregulation of genes and receptors involved in fructose metabolism and epigenetic predisposition [1,2].

Zingerone administered to rats orally during the neonatal period had programmed for long-term protection against the development of high-fructose diet-induced metabolic dysfunction, fatty liver and early features of nephropathy later in adult life of male and female rats. The mechanisms by which zingerone exerted these protective and beneficial effects could be attributed to its anti-obesity, hepatoprotective and nephroprotective properties secondary to its anti-oxidant and anti-inflammatory activities [3,4].

Glycaemic parameters, which could point to insulin resistance and hyperglycaemia, were not affected by the interventions of the current study despite the male and female rats had developed

lipid abnormalities. This could be attributed to the fact that development of obesity usually precedes type 2 diabetes [5].

The findings of this study indicated that long-term consumption of 20% fructose solution, as well as neonatal administration of zingerone did not adversely affect the red cell indices of the study rats. Interestingly, the current study observed a haematinic potential of zingerone. Except for the empty carcass mass and long bone densities that were heavier in female rats that consumed high-fructose diet, current study observed that both fructose and neonatal zingerone did not alter the GIT morphometry and extra growth parameters of the study animals. This implies that zingerone could be safe for oral administration in the neonatal period, despite reports that neonatal treatment with phytochemicals could cause toxicity as most physiological systems are not fully developed [6,7]. Thus, the findings of the current study indicated that administration of zingerone to neonates does not have long-term deleterious effect to the overall health status of the animals.

Additionally, the current study observed sexually dimorphic responses to the long-term consumption of high-fructose diets, in which female rats were more prone to fructose-induced metabolic derangements than their male counterparts. This could be due to increased fructose consumption observed with female rats in this study (Chapter 3).

Conclusively in order to reduce the global burden of fructose-induced metabolic disorders on health care delivery, a similar strategic use of zingerone in the critical period of developmental plasticity could be explored in humans to manage diet-induced metabolic syndrome; thereby replacing the use of animal models in exploring the therapeutic effects of plant phytochemicals. This could be in form of zingerone being incorporated as a supplement to infant food formula, which would be consumed orally during the neonatal period, and this could provide long-term protection against the development of diet-induced metabolic syndrome later in life.

8.2 Limitations and recommendations

Additional measures that were not investigated in this study, which could have further explored the long-term effects of fructose administration, as well as the potential effects of neonatal administration of zingerone should be considered in future studies. These include the following:

8.2.1 Further assessment of metabolic dysfunction

Hypertension, defined as abnormally elevated blood pressure, is one of the major components of metabolic syndrome [8], and was not evaluated in this study. Thus, I recommend future studies to

incorporate hypertension while exploring the fructose-induced metabolic dysfunction, and the potential therapeutic effects of neonatal zingerone, such that the full picture of metabolic syndrome could be explored.

Some metabolic hormones such as leptin and ghrelin also regulate metabolism, as they regulate satiety and β-cell development and functions [9,10]. These hormones were not evaluated in this study. Thus, I recommend future studies to evaluate leptin, ghrelin when investigating the long-term effects of high-fructose diets, as well as the therapeutic potential of neonatal administration of zingerone.

8.2.2 Further assessment of NAFLD

Obesity-induced oxidative stress is the major cause of NAFLD, in which there is imbalance between oxidant and anti-oxidant markers, in favour of oxidant markers [11]. Although the oxidant markers (MDA and TBARS) were determined, the antioxidant markers such as superoxide dismutase and glutathione could not be determined in the current study. Thus, I recommend future studies to evaluate these antioxidant enzymes in order to have a full picture of oxidative processes associated with fructose-induced metabolic dysfunction.

Despite some inflammatory markers (TNF-α and IL-6) were determined in this study, however, there is need for future studies to conduct immuno-histochemical staining of hepatocytes (which was not conducted in the current study) such that the presence of activated macrophages could be detected. By so doing, the extent of hepatic inflammatory processes could be evaluated.

8.2.3 Further assessment of nephropathy

Another limitation of this study is that, the kidney histological evaluation was done with light microscope instead of electron microscope, and renal tissue micro alterations could have been missed. Thus, future studies should consider using electron microscope as well as immuno-histochemical staining to histologically evaluate renal tissues.

8.2.4 Further assessment of general health status

8.2.4.1 Assessment of bones

Despite the fact that current study had determined the Seedor index of long bones, however, Seedor index only indicates the relative bone density [12]. Densitometry and dual-energy X-ray absorptiometry objectively measure bone density, and were not considered in the current study.

As such, I recommend future studies to objectively measure bone density using densitometry and dual-energy X-ray absorptiometry, the latter being the gold standard for measurement of bone density [13].

Despite it has been reported that consumption of excess fructose decreases the levels of calcium and phosphorous in the long bones of rats thereby affecting their bone breaking strength [14], Current study was unable to test for bone breaking strength. Thus, bone breaking strength tests should be considered in the future. In addition, the actual levels of calcium and phosphate should be determined from the ash content of the bones.

Osteocalcin is a hormone secreted by osteoblasts, and plays a critical role in bone metabolism and bone health [15]. Current study did not determine plasma osteocalcin. Thus, I recommend future studies to consider measuring plasma osteocalcin.

8.2.4.2 Histological assessment of pancreas and GIT

It has been shown that excess fructose consumption can adversely affect the GIT and pancreas [16], particularly through the development of non-alcoholic fatty pancreas disease (NAFPD) which is now being recognised as a major cause of morbidity and mortality globally [17]. NAFPD is the accumulation of fat in the pancreas, and is associated with visceral adiposity and metabolic syndrome [18]. Despite the pancreatic mass and GIT morphometry were not affected by the interventions of this study, histological evaluation of the GIT and pancreas was not done in the current study. This could have revealed micro-structural changes that could be associated with fructose consumption in rats. Thus, future studies should consider histomorphometry of these organs.

8.2.5 Assessment of molecular mechanism of action of fructose and zingerone

Excess dietary fructose consumption may directly alter the expression of genes and receptors involved carbohydrate and lipid metabolism [19]. These include PPARα, SREBP-1, ChREBP and TNF-α [20,21], which were not evaluated by the current study. The deleterious effects of fructose, and the beneficial effects of zingerone observed in this study could have affected the expression of these genes and receptors. Thus, I recommend the future studies to evaluate the expression of these genes, proteins and receptors (using quantitative real-time polymerase chain reaction and western blotting technique), as well as determination of DNA methylation and histone modification (epigenetic studies). This would provide a broader understanding of the molecular

mechanisms of action of fructose and zingerone that resulted in these long-term metabolic alterations and protective effects respectively, and the possible transgenerational benefits of the interventions.

The following section consists of appendices that are relevant to the current study.

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APPENDICES

Appendix 1: Plagiarism Declaration

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Nasiru Muhammad (Student number: 1332380) am a student registered for the degree of PhD in the academic year 2021.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature: _____ Date: 23-02-2021

Appendix 2: Animal Ethics Clearance Certificate

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2017/010/71/B

APPLICANT: Dr B Lembede

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: The potential of orally administered Zingerone to protect and to programme for protection against high-fructose high-cholesterol diet-induced metabolic derangements in Sprague-Dawley rats

Number and Species

50X 21 day old male and 50X 21 day old female Sprague-Dawley rats and 100X 4-day old male rats pups and 100X 4-day old female rat pups Sprague-Dawley rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 2017/10/31. This approval remains valid until 2019/11/12.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

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

Signed: _____ (Chairperson, AESC) Date: NOVEMBER 15, 2017

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

Signed: _____ (Registered Veterinarian) Date: 13 November 2017

cc: Supervisor: Professor E Chivandi and Professor K Erlwanger
Director: CAS

Works 2000/laln0015/AESCCert.wps

Appendix 3: First Modification of Ethics Clearance

AESC 2018 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 W. Lembede (A0026777)

b. School and email address: School of Physiology | Busisani.Lembede@wits.ac.za

c. Experiment to be modified / extended

		AESC NO	
Original AESC number	2017	010	71/B
Other M&Es :			

d. Project Title: The potential of orally administered Zingerone to protect and to programme for protection against high-fructose high-cholesterol diet-induced metabolic derangements in Sprague-Dawley rats

		No.	Species
e.	Number and species of animals originally approved:	300	Sprague Dawley
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:

- Include Assoc Prof Aletta Millen, Ms Nondumiso Immaculate Lushozi and Nasiru Muhammad as co-workers.
- Perform non-invasive cardiovascular measurements (echocardiography and aortic pulse wave velocity) on the rats at termination. The echocardiographic and aortic pulse wave velocity techniques have been performed in the CPGRU for many years and will be conducted by a single experienced operator. Rats will be anesthetized 15 minutes prior to the performance of echocardiography and aortic pulse wave velocity measurements.

j. Motivation for modification / extension:

- Ms Nondumiso Immaculate Lushozi and Nasiru Muhammad will be involved with animal husbandry, data collection and analysis and write-up of publications as part of their Masters and PhD studies respectively. Assoc Prof Aletta Millen will perform the cardiovascular measurements.
- The cardiovascular related measurements will provide insight on the effects of the interventions on the cardiovascular system of the rats.

Date: 4th of April 2018 Signature:

RECOMMENDATIONS

Date: 29/5/2018 Signature:

Chairman, AESC

Appendix 4: Second Modification of Ethics Clearance

AESC 2018 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 W. Lembede (A0026777)

b. School and email address: School of Physiology | Busisani.Lembede @wits.ac.za

c. Experiment to be modified / extended

		AESC NO	
Original AESC number	2017	010	71/B
Other M&Es :			1

d. Project Title: The potential of orally administered Zingerone to protect and to programme for protection against high-fructose high-cholesterol diet-induced metabolic derangements in Sprague-Dawley rats.

		No.	Species
e.	Number and species of animals originally approved:	300	Rats
f.	Number of additional animals previously allocated on M&Es:	0	
g.	Total number of animals allocated to the experiment to date:	134	
h.	Number of animals used to date:	134	

i. Specific modification / extension requested:

- To amend the title of the study.
- To amend the concentration of the fructose solution to be used in the study.
- To modify the study design so that experiment 2 will consist of 2 interventional stages (first and second) as against the previously approved 3 stages and consequently terminate the rats earlier than planned.
- To no longer use Dimethyl sulphoxide (DMSO) as a solvent for the zingerone.

j. Motivation for modification / extension:

Due to poor growth performance of rats noted by colleagues within our research lab who have used cholesterol supplementation, we would like to no longer use the cholesterol enriched feed but retain the fructose solution as originally planned. We have found fructose to be effective in inducing metabolic dysfunction as has been reported by others (Mamikutty et al., 2015). Additionally, our colleagues noted decreased fluid consumption when rats were given 25% w/v fructose solution. Thus I would like to use a lower concentration of 20% w/v fructose solution in the current study. We have previously found this (20%) concentration to be effective for metabolic studies.

I would like to change the focus of my study (second experiment) from adulthood to adolescence. There is a rising of prevalence of childhood obesity. Several studies have shown that feeding rats on a high-fructose diet from post-weaning for 6 weeks results in metabolic changes that are detectable in the adolescent period (Spahis et al., 2018). Thus a six week post weaning high fructose diet intervention should suffice for the purposes of my study. This

change in duration will necessitate that we remove the last phase of the originally approved study design. Instead of ending at 112 days the study will now be terminated at day 70. This will also result in less rats (80 instead of 200) being used in the study (See appendix 1).

DMSO is usually used as vehicle of delivering intervention in rats. Studies have shown that zingerone is sparingly soluble in water, and the oral absolute bioavailability is 72.2% (Archana and Maheswari, 2016; Hemalatha and Prince, 2014). Hence there is no need to use DMSO.

The proposed new title "The potential of orally administered Zingerone to protect and to programme for protection against high-fructose diet-induced metabolic derangements in Sprague-Dawley rats" will reflect the above mentioned modifications to the study design.

All other approved procedures will remain unchanged.

References:

Archana, A. K. & Maheswari, R. 2016. Zingerone ameliorates free radical scavengers and lipid profile of Wistar Albino rats. *International Journal of Pharma Sciences and Research,* 7, 254-260.

Hemalatha, K. L. & Prince, S. M. 2015. Antihyperlipidaemic, antihypertrophic, and reducing effects of zingerone on experimentally induced myocardial infarcted rats. *Journal of Biochemical and Molecular Toxicology,* 29, 182-188.

Mamikutty, N., Thent, Z. C. & Haji Suhaimi, F. 2015. Fructose-drinking water induced nonalcoholic fatty liver disease and ultrastructural alteration of hepatocyte mitochondria in male Wistar rat. *BioMed Research International,* 2015, 1-7.

Spahis, S., Alvarez, F., Ahmed, N., Dubois, J., Jalbout, R., Paganelli, M., Grzywacz, K., Delvin, E., Peretti, N. & Levy, Emile. 2018. Non-alcoholic fatty liver disease severity and metabolic complications in obese children: impact of omega-3 fatty acids. *The Journal of Nutritional Biochemistry,* 58, 28-36.

Date: 06/11/18 Signature: _____

RECOMMENDATIONS : AS LISTED BELOW

Date: 22/11/2018 Signature: _____ Chairman, AESC

ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

November 21, 2018

Certificate reference: 2017 010 71/B

Applicant : Busisani W Lembede
Department: Physiology
Tel: +27 (0)11 717 2154 ; **Email:** Busisani.Lembede@wits.ac.za

RE: Clarification/recommendations to M&E request

Title: The potential of orally administered Zingerone to protect and to programme for protection against high-fructose high-cholesterol diet-induced metabolic derangements in Sprague-Dawley rats.

Clarification:
The study is to be shortened – the end point is now going to be adolescence rather than late adulthood. The request is to continue with fructose solution straight after weaning rather than give a break before recommencing with fructose. Therefore the continual exposure to fructose without a break means that the study does not have to go on for so long.

Study numbers – 300 rats allocated & 134 used leaving 166 allocated but not used.
Expt 1 used 100 rats and the study has been completed
Expt 2 allocated 200 rats - 34 had been allocated to phase 2 at the time of application. The study is still in the early phase of gavaging as originally approved. M&E reduces study number to 80.

DMSO no longer required - warming in water to 41C solubilises the zingerone and hence DMSO is no longer required as a solvent.

- **APPROVED** November 21, 2018

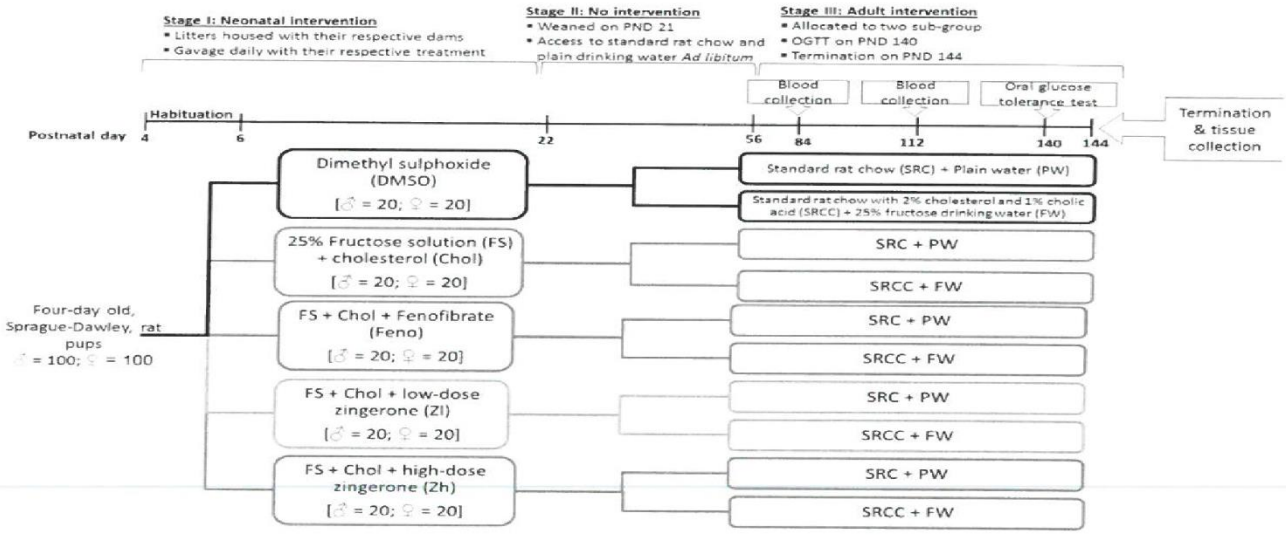
GP Candy

Geoffrey Candy
Chair : Animal Ethics Research Committee, University of the Witwatersrand

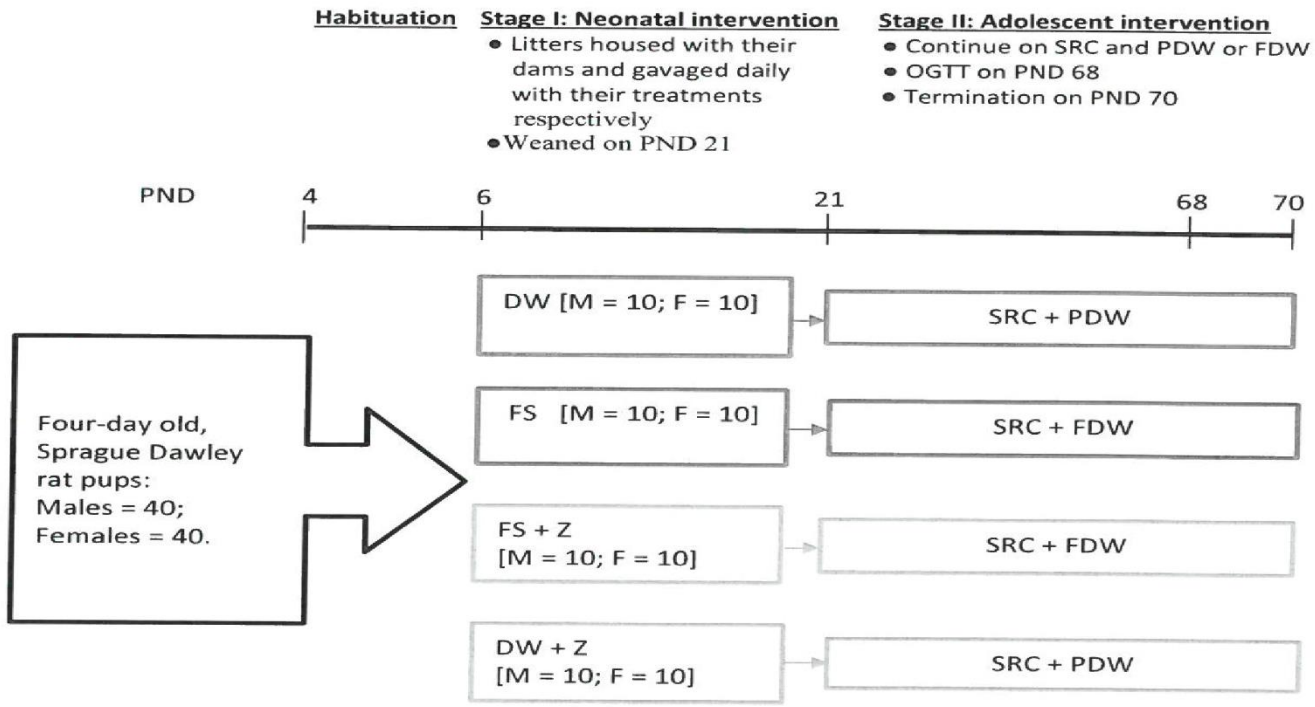
Geoffrey P Candy PhD
Professor, Department of Surgery, Faculty of Health Sciences, University of the Witwatersrand, *7 York Road Parktown 2193, Johannesburg; Tel: 27-11-717-2574; Email: geoffrey.candy@wits.ac.za*

Appendix 1

a. Timeline and study design for currently approved second experiment



b. Proposed timeline and study design for modification of second experiment



KEY: PND= postnatal day; DW= distilled water; FS= fructose solution; SRC= standard rat chow; PDW= plain drinking water; OGTT= oral glucose tolerance test; FDW= fructose drinking water; Z= zingerone; M = males; F = females.

Appendix 5: Third Modification of Ethics Clearance

AESC 2012 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 W. Lembede (A0026777)

b. Department: School of Physiology/ busisani.lembede@wits.ac.za

c. Experiment to be modified / extended

Original AESC number	**2017**	**010**	**71/B**
Other M&Es :			2

AESC NO

d. Project Title: The potential of orally administered Zingerone to protect and to programme for protection against high-fructose diet-induced metabolic derangements in Sprague-Dawley rats.

		No.	**Species**
e.	Number and species of animals originally approved:	180	Rats
f.	Number of additional animals previously allocated on M&Es:	0	
g.	Total number of animals allocated to the experiment to date:	180	Rats
h.	Number of animals used to date:	179	Rats

i. Specific modification / extension requested:

- To include the following students as co-workers:

Student number	Name & Surname	Reason
2246651	Ajah Austin Azubuike	Provide technical assistance and animal husbandry
1889383	Bernice Asiedu	Provide technical assistance and animal husbandry
1965524	Abubakar Namadina Muhammad	Provide technical assistance and animal husbandry
1427369	Nonosi Kennedi	Will work on the liver samples and plasma to investigate NAFLD as part of her BHSc honours degree requirements
1157915	Tholane Tsotetsi	Provide technical assistance and animal husbandry
361160	Sthembi Notha	Provide technical assistance and animal husbandry

j. Motivation for modification / extension:

Ajah Austin Azubuike, Bernice Asiedu, Abubakar Namadina Muhammad, Tholane Tsotetsi, and Sthembi Notha will Provide technical support during the course of the study. They will be involved with the animal husbandry and data collection. While Nonosi Kennedi will work on the liver samples and plasma to investigate non-alcoholic fatty liver disease (NAFLD) as part of her BHSc honours degree requirements.

All other approved protocols will remain unchanged.

AESC 2012 M&E

Date:27/03/19 Signature: 

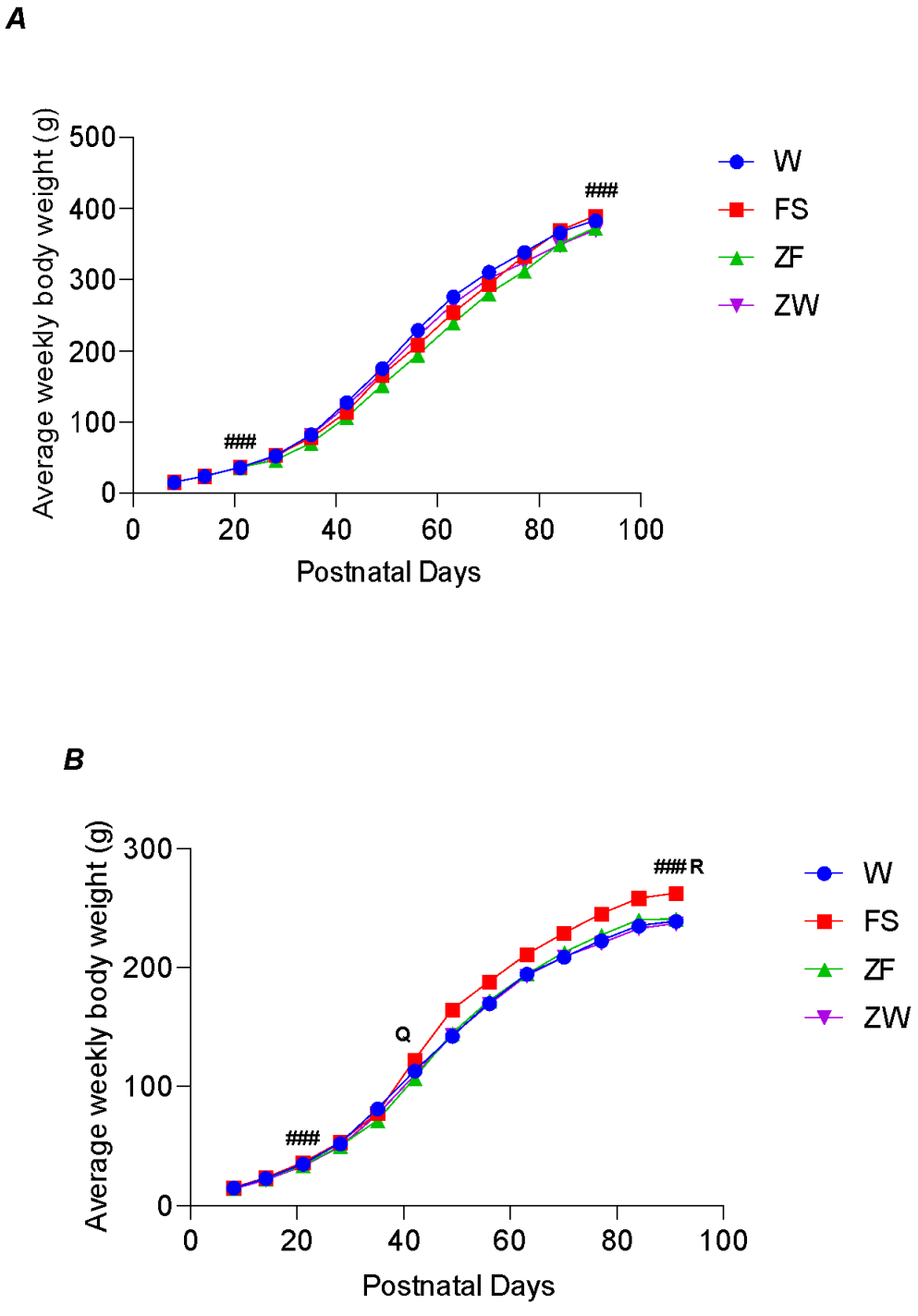
RECOMMENDATIONS

Addition of Ajah Austin Azubuike, Bernice Asiedu, Abubakar Namadina Muhammad, Tholane Tsotetsi, Sthembi Notha and Nonosi Kennedi – condition: They have to complete the CAS orientation course

Date: 28/02/2019 Signature: 

Chairman, AESC pp.

Appendix 6: Supplementary Figure S1 (For chapter 3)



Supplementary Fig S1: Effects of neonatally administered zingerone on average weekly body weight of high-fructose diet fed male (*A*) and female (*B*) rats in adulthood.

Data expressed as mean ± standard deviation. ### = significant increase in body mass from induction (PND 8) to weaning (PND 21) and from weaning to termination (PND 91) at P < 0.0001; Q = postnatal day 42 at which the weekly body weight of the group FS started to differ compared to the other groups in the female rats; R = postnatal day 91 at which the terminal body weight of the group FS was significantly different at P < 0.0001 compared to the other groups in the female rats; W = gavaged with 10 ml/kg body weight (bwt) of distilled water before weaning and then provided unlimited access to plain tap water post-weaning [Males (M) = 9, Females (F) = 11]; FS = gavaged with 10 ml/kg bwt of a 20% fructose solution before weaning and then provided unlimited access to a 20% fructose solution to drink post-weaning (M = 9, F = 11); ZF = gavaged with a combination of 20% fructose solution (10 ml/kg bwt) and zingerone (40 mg/kg bwt) before weaning and then unlimited access to 20% fructose solution to drink post-weaning (M = 9, F = 12); ZW = gavaged with zingerone only at 40 mg/kg bwt dissolved in distilled water before weaning and given unlimited access to plain tap water to drink post-weaning (M = 8, F = 10).

Appendix 7: Supplementary Table S1 (For chapter 3)

Supplementary Table S1: Effects of neonatally administered zingerone on weekly food (*A*), fluid (*B*) and calorie intake (*C*) of high-fructose diet fed rats in adulthood

A

Experimental Week	Sex	Weekly food intake (g/100g body mass)			
		W	FS	ZF	ZW
9	Male	53.00 ± 1.60^{a}	32.00 ± 4.80^{b}	32.00 ± 5.20^{b}	55.00 ± 0.84^{a}
	Female	$57.00 \pm 2.60^{a\lambda}$	$32.00 \pm 4.60^{b\lambda}$	$34.00 \pm 5.50^{b\lambda}$	$55.00 \pm 2.20^{a\lambda}$
10	Male	45.00 ± 0.55^{a}	30.00 ± 5.30^{b}	32.00 ± 3.20^{b}	47.00 ± 2.10^{a}
	Female	$55.00 \pm 1.50^{a\lambda}$	$29.00 \pm 2.80^{b\lambda}$	$32.00 \pm 6.00^{b\lambda}$	$54.00 \pm 4.60^{a\lambda}$
11	Male	45.00 ± 1.20^{a}	28.00 ± 6.80^{b}	28.00 ± 2.70^{b}	45.00 ± 0.11^{a}
	Female	$49.00 \pm 3.90^{a\lambda}$	$24.00 \pm 2.30^{b\lambda}$	$25.00 \pm 3.50^{b\lambda}$	$49.00 \pm 3.30^{a\lambda}$
12	Male	43.00 ± 2.20^{a}	22.00 ± 5.90^{b}	25.00 ± 4.40^{b}	47.00 ± 4.60^{a}
	Female	$52.00 \pm 5.10^{a\lambda}$	$25.00 \pm 8.60^{b\lambda}$	$20.00 \pm 3.60^{b\lambda}$	$54.00 \pm 2.10^{a\lambda}$

B

Experimental Week	Sex	Weekly fluid intake (ml/100g body mass)			
		W	FS	ZF	ZW
9	Male	52.00 ± 0.11^{a}	84.00 ± 18.00^{b}	80.00 ± 14.00^{b}	47.00 ± 4.10^{a}
	Female	$84.00 \pm 9.70^{a\lambda}$	$110.00 \pm 9.00^{b\lambda}$	$110.00 \pm 14.00^{b\lambda}$	$79.00 \pm 11.00^{a\lambda}$
10	Male	59.00 ± 5.50^{a}	100.00 ± 13.00^{b}	95.00 ± 6.40^{b}	61.00 ± 4.60^{a}
	Female	$107.00 \pm 9.20^{a\lambda}$	$138.00 \pm 7.50^{b\lambda}$	$149.00 \pm 23.00^{b\lambda}$	$100.00 \pm 9.20^{a\lambda}$
11	Male	60.00 ± 8.20^{a}	94.00 ± 3.50^{b}	90.00 ± 1.90^{b}	66.00 ± 19.00^{a}
	Female	$93.00 \pm 16.00^{a\lambda}$	$144.00 \pm 12.00^{b\lambda}$	$133.00 \pm 11.00^{b\lambda}$	$85.00 \pm 8.70^{a\lambda}$
12	Male	62.00 ± 3.40^{a}	97.00 ± 8.40^{b}	99.00 ± 9.90^{b}	58.00 ± 1.70^{a}
	Female	$92.00 \pm 8.30^{a\lambda}$	$132.00 \pm 13.00^{b\lambda}$	$141.00 \pm 15.00^{b\lambda}$	$89.00 \pm 15.00^{a\lambda}$

C

Experimental Week	Sex	Total weekly calories intake (Kcal/100g body mass)			
		W	FS	ZF	ZW
9	Male	120.96 ± 4.37^{a}	155.87 ± 8.12^{b}	154.23 ± 19.05^{b}	131.92 ± 1.61^{a}
	Female	$158.96 \pm 7.16^{a\lambda}$	$173.77 \pm 1.07^{b\lambda}$	$171.79 \pm 5.69^{b\lambda}$	$154.86 \pm 6.08^{a\lambda}$
10	Male	119.41 ± 4.39^{a}	164.84 ± 6.98^{b}	160.92 ± 7.50^{b}	131.15 ± 5.93^{a}
	Female	$153.86 \pm 4.16^{a\lambda}$	$183.34 \pm 14.73^{b\lambda}$	$203.48 \pm 18.61^{b\lambda}$	$151.60 \pm 12.97^{a\lambda}$
11	Male	115.38 ± 10.38^{a}	152.46 ± 16.57^{b}	142.65 ± 14.07^{b}	125.89 ± 0.30^{a}
	Female	$136.78 \pm 10.99^{a\lambda}$	$183.35 \pm 4.95^{b\lambda}$	$165.14 \pm 3.36^{b\lambda}$	$136.38 \pm 9.17^{a\lambda}$
12	Male	120.27 ± 6.02^{a}	138.29 ± 13.44^{a}	147.98 ± 18.96^{b}	131.48 ± 12.98^{a}
	Female	$145.60 \pm 14.31^{a\lambda}$	$175.65 \pm 28.46^{b\lambda}$	$167.84 \pm 19.13^{b\lambda}$	$151.54 \pm 6.00^{a\lambda}$

Data expressed as mean ± standard deviation. ^{ab} = within row means with different letters significantly different at $P < 0.05$; $^{\lambda}$ = female rats significantly had higher food, fluid or calorie intake at $P < 0.05$; W = gavaged with 10 ml/kg body weight (bwt) of distilled water before weaning and then provided unlimited access to plain tap water post-weaning [Males (M) = 9, Females (F) = 11]; FS = gavaged with 10 ml/kg bwt of a 20% fructose solution before weaning and then provided unlimited access to a 20% fructose solution to drink post-weaning (M = 9, F = 11); ZF = gavaged with a combination of 20% fructose solution (10 ml/kg bwt) and zingerone (40 mg/kg bwt) before weaning and then unlimited access to 20% fructose solution to drink post-weaning (M = 9, F = 12); ZW = gavaged with zingerone only at 40 mg/kg bwt dissolved in distilled water before weaning and given unlimited access to plain tap water to drink post-weaning (M = 8, F = 10).

Appendix 8: Soxhlet Procedure for Lipid Extraction

1. Set the Soxhlet apparatus
2. Soak the extraction thimble into the petroleum ether
3. Place a fat-free cotton wool into the thimble
4. Accurately weigh 0.5 g of the freez-dried ground liver sample with a spatula, and note the weight
5. Put the weighed sample into the thimble above the cotton wool, and place the thimble into the Soxhlet extraction chamber
6. Weigh the empty distillation flask (round bottom flask) and note the weight
7. Add 200 ml of petroleum ether into the distillation flask
8. Place the distillation flask (containing the petroleum ether) onto the heating pad
9. Set the thermostat at 50 ± 10°C (boiling point of petroleum ether)
10. Turn on the cooling water supply to the condensers
11. Switch on the power supply
12. Run the procedure for 2 hours
13. Switch off the power supply and turn off the water supply
14. Remove the thimble from the extractor, and pour the remaining ether into the distillation flask
15. Distillate the petroleum ether on an evaporator at 50 ± 10°C until all the ether evaporates leaving only the oil in the flask
16. Allow the flask to cool before weighing
17. Weigh the flask containing the oil, and note the weight

Calculation of the Extracted Fat (%):

$$\% \text{ fat} = \frac{\text{Weight of flask with oil} - \text{weight of empty flask}}{\text{Weight of liver sample}} \times 100$$

Appendix 9: Rat Enzyme-Linked Immunosorbent Assay (ELISA) Kit Protocol

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 is recommended that all samples and standards be assayed in duplicate.

1. **Add Sample:** Add 100μL 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℃.
2. **Biotinylated Detection Ab:** Remove the liquid of each well, don't wash. Immediately add 100 μL 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 μL) (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 μL 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 μL 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 colour change, but not more than 30 minutes. When apparent gradient appeared in standard wells, user should terminate the reaction.
7. **Stop**: Add 50 μL of Stop Solution to each well. Then, the colour 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.
10. **Calculation of results:**
    - Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density.
    - Create a standard curve by plotting the mean OD value for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph.
    - It is recommended to use some professional software to do this calculation, such as curve expert 1.3 or 1.4. In the software interface, a best fitting equation of standard curve will be calculated using OD values and concentrations of standard sample. The software will calculate the concentration of samples after entering the OD value of samples.

Appendix 10: IDEXX Catalyst Dx Chemistry Analysis Protocol

The procedure is conducted on two separate machines connected to each other: the IDEXX Vetlab Computer and the Catalyst Dx Chemistry Analyser.

On the IDEXX VetLab Computer Screen:

1. Click “Analyse sample”
2. Enter the patient/client’s information
3. Select the analysis you wish to run
4. The patient/client’s information is automatically transferred to the Catalyst Dx Chemistry Analyser.

On the Catalyst Dx Chemistry Analyser:

1. Select the patient/client from the touch screen
2. Choose the sample type you wish to run
3. Load the pipettes into the analyser
4. Load the chemistry slide (cassette) into the analyser
5. Sample (whole blood/plasma/serum) would have been exposed to room temperature
6. Mix the sample gently by inverting the microtube
7. Load 200 µl of the sample into the analyser
8. Press “Run”
9. The result appears in 5-8 minutes, and automatically transfer to the IDEXX VetLab computer for printing and sharing.

Appendix 11: Published Manuscript (Chapter 3)

Journal of Developmental Origins of Health and Disease

www.cambridge.org/doh

Neonatal zingerone protects against the development of high-fructose diet-induced metabolic syndrome in adult Sprague-Dawley rats

N. Muhammad[1,2], B. W. Lembede[1] and K. H Erlwanger[1]

[1]School of Physiology, Faculty of Health Sciences, University of Witwatersrand, Johannesburg, South Africa and [2]Department of Physiology, College of Health Sciences, Federal University Birnin Kebbi, Birnin Kebbi, Nigeria

Original Article

Cite this article: Muhammad N, Lembede BW, and Erlwanger KH. Neonatal zingerone protects against the development of high-fructose diet-induced metabolic syndrome in adult Sprague-Dawley rats. *Journal of Developmental Origins of Health and Disease* doi: 10.1017/S2040174420000525

Received: 5 December 2019
Revised: 8 May 2020
Accepted: 11 May 2020

Keywords:
Fructose; metabolic dysfunction; zingerone; neonatal; dyslipidaemia; obesity

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Abstract

During the early postnatal period, dietary manipulations can alter the developmental trajectory of the growing offspring, causing beneficial or adverse health outcomes later in adult life. We investigated the potential preventive effects of neonatal zingerone intake on the development of fructose-induced metabolic derangements in rats.

Four-day old male and female Sprague-Dawley rat pups ($n = 79$) were randomly grouped and administered: 10 ml/kg body weight (bwt) of distilled water (W), 10 ml/kg bwt 20% fructose solution (FS), 10 ml/kg bwt fructose solution + 40 mg/kg bwt of zingerone in distilled water (ZF) or 40 mg/kg bwt of zingerone in distilled water (ZW) pre-weaning. After weaning, W and ZW continued on unlimited tap water, while FS and ZF continued on unlimited fructose solution for 10 weeks. Body mass and food and fluid intake were evaluated, plasma was collected for metabolic assays and visceral fat was quantified.

Food intake was decreased, fructose and overall caloric intake were increased due to fructose feeding in both sexes ($P < 0.05$). When compared with the controls, the high-fructose diet significantly raised the terminal body masses of females ($P < 0.0001$), concentrations of triglycerides, total cholesterol, LDL-c, TG:HDL-c ratio and visceral fat mass relative to bwt in both sexes ($P < 0.05$). Zingerone prevented ($P < 0.05$) the fructose-induced increase in body mass (females) and hypercholesterolemia (both sexes). Levels of HDL-c, glycaemic parameters and adiponectin were not affected by the interventions ($P > 0.05$). Sex-related differences were observed in food, fluid and caloric intake, terminal mass, cholesterol subtypes and visceral fat percentage ($P < 0.05$).

Zingerone could be used strategically in the neonatal phase as a prophylatic management of high-fructose diet-induced metabolic syndrome.

Introduction

The early postnatal period of life is a dynamic developmental period where dietary and environmental interventions can affect an individual's phenotypic physiology, leading to beneficial or adverse health outcomes in subsequent stages of life.[1] Nutrients and phytochemicals can interact with the epigenetic machinery of the individual during the neonatal period leading to epigenetic changes.[2] These epigenetic changes are associated with dysregulation of gene expression that underlies the developmental programming of adverse health outcomes in adulthood.[3] The diet-induced epigenetic changes occurring during the neonatal period can also affect the individual's immature brain.[3]

During the early postnatal period, the differentiating satiety centre in the brain is influenced by intrinsic and extrinsic factors that affect appetite, and consequently, nutrition and weight gain cannot be adequately regulated.[4] The dietary nutrients that can have an effect during the neonatal period include high-fructose diets.[5] Excess dietary fructose consumption during childhood leads to the development of cardio-metabolic disorders including central obesity, dyslipidaemia, type-II-diabetes and coronary heart disease in the subsequent developmental periods of life.[6] The aforementioned are amongst the components of metabolic syndrome.[7]

Over one billion people in the world are affected by metabolic syndrome, with females having a higher prevalence (53%) than males (43%), while children account for 3–4%.[8,9] This high prevalence of metabolic syndrome is associated with an increase in fructose consumption among other factors.[10]

Most of the ingested fructose is transported to the liver where it is metabolised leading to visceral fat deposition, dyslipidaemia, obesity, excessive weight gain, insulin resistance and type-II-diabetes.[11] Whilst glucose metabolism is regulated at the level of phosphofructokinase, by the amount of adenosine triphosphate (ATP) generated and insulin secreted, fructose

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metabolism is independent of these two factors.[11] This results in fructose being converted to fatty acids, triglycerides and low-density lipoprotein cholesterol (LDL-c) which predisposes to the pathogenesis of obesity and metabolic syndrome.[11] Fructose also suppresses hepatic fatty acid oxidation and high-density lipoprotein cholesterol secretion leading to visceral adiposity and an increase in the overall body mass.[12]

Fructose-rich diets affect insulin sensitivity via lipogenesis leading to insulin resistance and eventually type-II-diabetes.[13] Adiponectin, an anti-inflammatory adipokine, enhances insulin sensitivity, energy metabolism and fatty acid oxidation by activating peroxisome proliferator-activated receptor alpha (PPARα).[14] Fructose decreases adiponectin signalling leading to hypoadiponectinaemia, which may affect the extent to which adiponectin inhibits lipogenesis thereby predisposing individuals to visceral obesity and metabolic dysfunction in later life.[14]

Management of metabolic disorders remains a challenge globally due to the expense of synthetic conventional drugs and poor healthcare facilities, especially in developing countries, making the drugs inaccessible to most.[15] The currently used drugs also tend to be monotherapeutic, targeting specific components of the metabolic syndrome and hence patients must take several formulations simultaneously and chronically to treat the conditions, resulting in adverse side effects, drug reactions, non compliance and increased costs.[16,17] Given these challenges, there is an increasing interest in the strategic use of medicinal plants and naturally derived phytochemicals with a broad spectrum of biological action.[17] Previous studies have demonstrated positive health outcomes with the strategic use, during the neonatal period, of phytochemicals including S-allyl cysteine,[18] oleanolic acid[18] and ursolic acid[19] as protection against metabolic dysfunction.

Zingerone (Vanillyl acetone) is a bioactive ketone found in ginger (*Zingiber officinale*) and is used as a flavouring agent in the food industry.[20] It has demonstrated antiobesity (fructose-induced), hypolipidaemic (fructose-induced) and antidiabetic (alloxan and streptozotocin-induced) properties in adult male rats.[21–25] The effects of zingerone on the liver include increase in PPAR activities and fatty acid oxidation that attenuate lipogenesis and preventing hepatic damage via inhibition of free radicals generation, downregulating inflammatory cytokines[25,26] and decreasing the activity of ALT[26,27] (our target for studies). The biological properties of zingerone make it a possible candidate that could be administered during the neonatal period to protect against long-term adverse metabolic outcomes secondary to high-fructose diets.

Metabolic studies were previously mostly conducted on adult males.[10,24,28,29] However, components of metabolic syndrome are also observed in growing children, with sexually dimorphic features between males and females.[3] We thus explored the potential of neonatal orally administered zingerone to programme for long-term protection against the adverse metabolic outcomes secondary to a high-fructose diet in male and female growing rats.

Methods

Study setting

The study was undertaken at the Central Animal Service of the University of Witwatersrand, Johannesburg, South Africa, following approval by the Animal Research Ethics Committee of the University of Witwatersrand (Reference No: 2017/010/71/B). The study was conducted according to internationally accepted principles for laboratory animal use and care as stipulated in South African National Standard (SANS 10386:2008) and Animals Protection Act, 1962: Act No. 71. The manuscript was compiled in alignment with the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines.

Animal housing

The experiment used 4-day-old male and female rat pups and consisted of a pre-weaning stage (postnatal days 8–21) and a post-weaning stage (postnatal days 22–91). The pre-weaning stage involved housing of the pups in each litter with their dams, respectively, in Perspex cages that were lined with wood shavings. In the post-weaning stage, the pups were housed singly in Perspex cages. In both stages, the room of the animals was kept at a temperature range of 24–26 °C with a 12-h dark and light cycles, with lights on from 7:00 h to 19:00 h for all the stages of the experiment.

Study design

A total of 79, 4-day-old suckling male and female Sprague-Dawley rat pups weighing 9.7 ± 1.1 g were received from seven nulliparous dams, each with a litter consisting of 8–12 pups. The sample size ranged between 8 and 12 rats per group and was calculated based on a previous study conducted in our research Laboratory[16] using the following formula:[30]

$$\text{Sample size} = 2\ SD^2\ (1.96 + 0.842)^2/d^2$$

where SD = standard deviation from previous study; 1.96 = type 1 error of 5%; 0.842 = at 80% power; d = difference between mean values.

The pups were acclimatised to handling and the environment for 4 days and then randomly allocated to four treatment groups: Group I which served as the negative control (W) [gavaged with 10 ml/kg body weight (bwt) of distilled water before weaning and then provided unlimited access to plain tap water post-weaning, $n = 20$; M = 9, F = 11]; Group II (FS) received fructose only (gavaged with 10 ml/kg bwt of a 20% fructose solution before weaning and then provided unlimited access to a 20% fructose solution to drink post-weaning, $n = 20$; M = 9, F = 11) to induce metabolic dysfunction; Group III was administered zingerone (40 mg/kg bwt) neonatally to programme for protection against long-term high-fructose consumption, received 20% fructose solution (10 ml/kg bwt) neonatally and then unlimited access to 20% fructose solution to drink post-weaning (ZF, $n = 21$; M = 9, F = 12); Group IV administered with zingerone only (40 mg/kg bwt of zingerone dissolved in distilled water before weaning and given unlimited access to plain tap water to drink post-weaning (ZW, $n = 18$, M = 8, F = 10) to assess the effects of zingerone alone on metabolic health of the animals. In addition, all the pups nursed freely from their respective dams during the pre-weaning stage and later had free access to standard rat chow [Labchef Rodent Breeder, Nutritionalhub (Pty) Ltd, Stellenbosch 7602, South Africa] during the post-weaning stage.

Fructose (Natures Choice, South Africa) was made as a 20% solution by diluting 200 g of fructose in one litre of water.[29] Food grade zingerone (W312401-1KG) was purchased from Sigma-Aldrich (USA).

Measurements

Food, fluid and calorie intake

The food and fluid intake by each rat was determined weekly in the last 4 weeks of intervention. At the end of each week, the remaining amounts of food in the cage were subtracted from the total amounts supplied at the beginning of the week. The fluid was provided in freshly prepared predetermined amounts such that there was a constant supply. The remaining fluid was then subtracted from the total supplied at the end of each week. The weekly food and fluid consumption were computed as a percentage of body mass as g/100 g and ml/100 g, respectively.

The total weekly calorie intake was determined by multiplying the amount of food and fructose consumed each week by their respective reference calorie values, and the two were then added together.

Body mass

The rats were weighed daily during the pre-weaning stage and then twice a week during the post-weaning stage by individually placing the rats in a pre-weighed cage on an electronic balance (Snowrex Electronic Scale, Clover Scales, Johannesburg).

Terminal procedures

On postnatal day 90, the rats were fasted from solid feed but provided plain drinking water overnight for 12 h. Terminal body mass was then determined (on postnatal day 91) as stated earlier. In order to quantify fasting blood glucose, a drop of blood was taken after a pinprick on the tail vein. The fasting concentration of glucose in the blood was quantified using a glucose meter (Contour Plus Bayer Health Care, Diabetes Care, Isando, South Africa) according to the manufacturer's instructions. The rats were then euthanised with sodium pentobarbitone (Centaur Laboratories, Johannesburg, South Africa) at 150 mg/kg body weight intraperitoneally, which is considered as appropriate and acceptable euthanasia in rats.[31]

A ventral midline incision was made on the thorax and abdomen, and intra-cardiac blood was drawn into syringes via attached needles. The blood samples were then transferred into heparinised tubes (BD Vacutainer, Plymouth, UK) and centrifuged (Rotofix 32A, Hettich Zentrifugen, Germany) at 3700 *g* for 15 min at 20 °C. The plasma was harvested and stored at −20 °C for later assays.

Visceral fat (surrounding the liver, kidneys, pancreas, stomach, small and large intestines) was carefully removed and weighed on a Presica 310M digital scale (Precision Instruments, Johannesburg, South Africa).

Blood parameters

Lipid profile

The stored plasma samples were thawed and triglycerides (TGs), total cholesterol (TC), LDL-c and high-density lipoprotein cholesterol (HDL-c) concentrations were determined using specific enzyme-based assay kits (Elabscience Biotechnology Co., Ltd, Wuhan, Hubei, China).

Adiponectin, insulin and Homeostasis Model of Assessment

Fasting plasma insulin and adiponectin were determined using rat-specific insulin and adiponectin Enzyme-Linked Immuno-Sorbent Assay kits (Elabscience Biotechnology Co., Ltd, Wuhan, Hubei, China). The insulin resistance index was computed according to the Homeostasis Model of Assessment (HOMA-IR):[32]

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

Statistical analysis

GraphPad Prism 5 software (Graph-Pad Software Inc., San Diego, USA) was used to analyse the data from all the animals. Data were expressed as mean ± standard deviation. A one-way analysis of variance [(ANOVA), parametric] was used to analyse multiple-group data on food, fluid and calorie intake, blood parameters and visceral fat, while repeated-measures ANOVA was used to analyse within-group data on weekly body mass, food and fluid intake. The multiple-comparisons Tukey *post hoc* test was used to compare the means. A two-way ANOVA was used to compare the effect of variation in treatment, sex and interaction, and statistical significance was considered when $P < 0.05$.

Results

Food, fluid and calorie intake in adulthood

The weekly food, fluid and calorie intake of the rats in the last 4 weeks of the intervention period is shown in Supplementary Tables S1*A*, S1*B* and S1*C*, respectively. Rats that received fructose with or without zingerone (FS; ZF) had significantly [$P = 0.0005$ (Males); $P < 0.0001$ (Females)] lower food intake compared to the negative controls (W) and the group administered zingerone only neonatally (ZW; Supplementary Table S1*A*). There was no difference [$P = 0.7618$ (Males); $P = 0.1083$ (Females)] in the food intake between the negative controls (W) and the group that received zingerone only (ZW), as well as between the fructose only group (FS) and the group that had fructose and zingerone (ZF) [$P = 0.6511$ (Males); $P = 0.5234$ (Females); Supplementary Table S1*A*].

Rats that received fructose with or without zingerone (FS; ZF) had significantly [$P = 0.0005$ (Males); $P < 0.0001$ (Females)] higher fluid intake compared to the controls (W) and the group that had zingerone only (ZW; Supplementary Table S1*B*). There were no differences [$P = 0.4529$ (Males); $P = 0.3105$ (Females)] in the fluid intake between the control (W) and zingerone only (ZW) groups, as well as between the fructose only group (FS) and the group administered a combination of fructose and zingerone (ZF) [$P = 0.3563$ (Males); $P = 0.1350$ (Females); Supplementary Table S1*B*).

Rats that received fructose with or without zingerone (FS; ZF) had significantly [$P = 0.0003$ (Males); $P < 0.0001$ (Females)] higher caloric intake compared to the controls (W) and the group that had zingerone only (ZW; Supplementary Table S1*C*). There were no differences [$P = 0.2802$ (Males); $P = 0.1113$ (Females)] in the calorie intake between the control (W) and zingerone only (ZW) groups, as well as between the fructose only group (FS) and the group administered a combination of fructose and zingerone (ZF) [$P = 0.8165$ (Males); $P = 0.9342$ (Females); Supplementary Table S1*C*].

However, female rats had significantly higher food [$P = 0.0002$ (sex effect), $P < 0.0001$ (treatment effect), $P = 0.0028$ (interaction effect)], fluid [$P < 0.0001$ (sex, treatment and interaction effects)] and calorie [$P < 0.0001$ (sex and treatment effects), $P = 0.0015$ (interaction effect)] intake in comparison to their male counterparts.

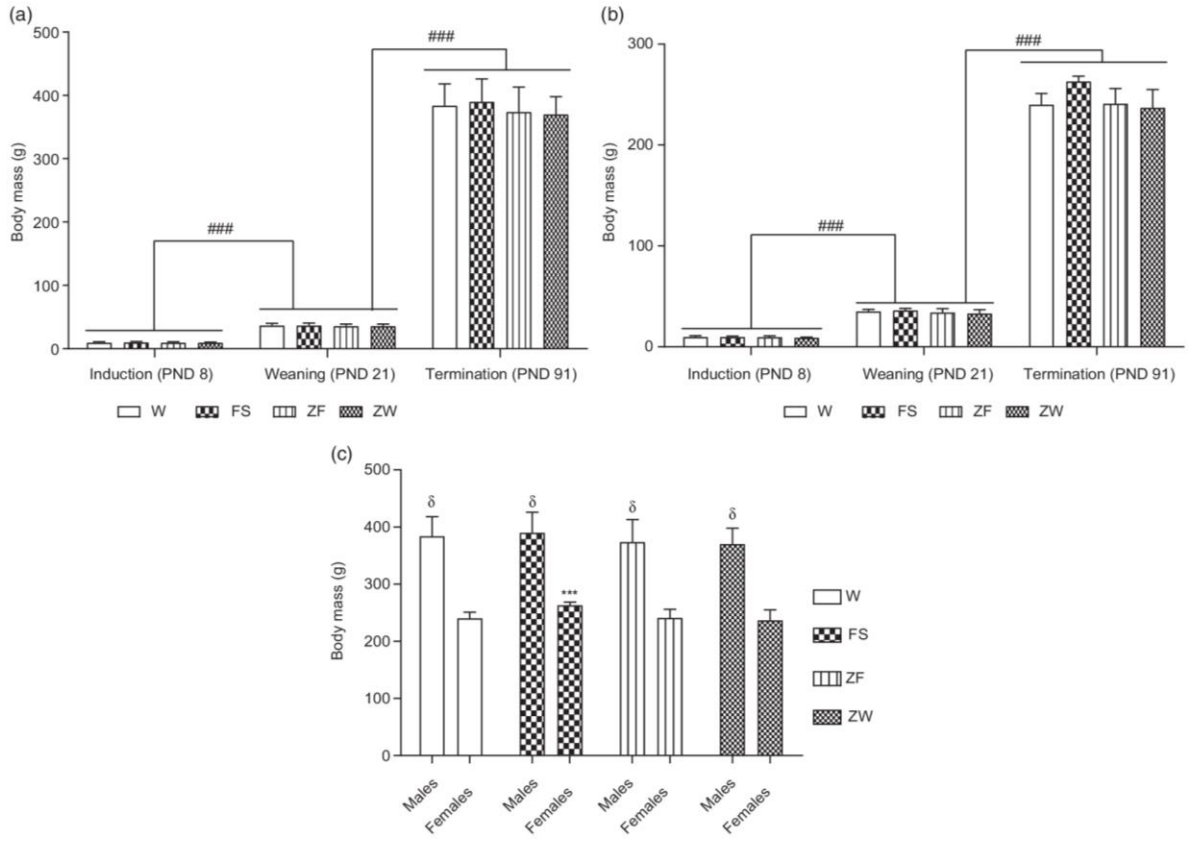

Fig. 1. Effects of neonatally administered zingerone on average body mass of high-fructose fed male (a), and female (b) rats from induction to weaning and termination, and compared terminal mass between males and females (c). Data expressed as mean ± standard deviation. ### = $P < 0.0001$ when induction is compared to weaning and when weaning is compared to termination; *** = $P < 0.0001$ when FS termination mass is compared to other treatments in females; δ = $P < 0.0001$ when males' termination masses are compared to those of females; W = gavaged with 10 ml/kg body weight (bwt) of distilled water before weaning and then provided unlimited access to plain tap water post-weaning (M = 9, F = 11); FS = gavaged with 10 ml/kg bwt of a 20% fructose solution before weaning and then provided unlimited access to a 20% fructose solution to drink post-weaning (M = 9, F = 11); ZF = gavaged with a combination of 20% 3 fructose solution (10 ml/kg bwt) and zingerone (40 mg/kg bwt) before weaning and then unlimited access to 20% fructose solution to drink post-weaning (M = 9, F = 12); ZW = gavaged with zingerone only at 40 mg/kg bwt dissolved in distilled water before weaning and given unlimited access to plain tap water to drink post-weaning (M = 8, F = 10); PND = postnatal day; M = males; F = females.

Body mass

The induction, weaning and terminal body masses of the study rats are represented in Fig. 1. All the rats grew significantly ($P < 0.0001$) from induction to weaning as well as from weaning to termination. In males, there were no differences in the induction ($P = 0.5976$), weaning ($P = 0.9658$) and terminal ($P = 0.6214$) body masses of the rats across treatment groups (Fig. 1a).

In females, there were no differences in the induction ($P = 0.3127$) and weaning ($P = 0.1456$) body masses of the rats across treatment groups (Fig. 1b). However, the high-fructose diet significantly ($P < 0.0001$; FS vs other treatments) increased the terminal body mass of the female rats. Thus, neonatal administration of zingerone prevented the high-fructose diet-induced increase in terminal body mass in female rats. The body weight data showing the longitudinal weight curves at time points in which changes in body weight occurred in male and female rats are shown in Supplementary Figure S1*A* and *B*, respectively.

Despite no difference in the induction [$P = 0.1931$ (sex effect), $P = 0.4517$ (treatment effect), $P = 0.7884$ (interaction effect)] and weaning [$P = 0.0640$ (sex effect), $P = 0.1933$ (treatment effect), $P = 0.8121$ (interaction effect)] body masses between male and female rats, the male rats had significantly [$P < 0.0001$ (sex effect), $P = 0.0310$ (treatment effect), $P = 0.7537$ (interaction effect) higher terminal body masses than females (Fig. 1c).

Clinical metabolic parameters in circulation

Table 1 shows the plasma lipid profile of the study rats. In both males and females, the high-fructose diet (FS) significantly increased plasma triglyceride concentrations [$P = 0.0226$ (males);$P = 0.0049$ (females)], triglycerides to HDL-c ratio [$P = 0.0190$ (males); $P = 0.0084$ (females)], concentrations of TC [$P = 0.0970$ (males); $P = 0.0005$ (females)] and LDL-c [$P = 0.0002$ (males); $P < 0.0001$ (females)] compared to the control (W; Table 1). The high-fructose diet-induced hypercholesterolemia (both sexes), hyper-LDL-cholesterolaemia (both sexes) and increase in triglycerides to HDL-c ratio (females only) were prevented ($P > 0.05$ when ZF was compared to control) by the neonatal orally administered zingerone (Table 1; with zingerone treated groups having similar concentrations of TC, LDL-c and triglycerides to HDL-c ratio with the control). The concentrations

Table 1. Effects of neonatally administered zingerone on lipid profile of high-fructose fed rats in adulthood

Parameter	Sex	W	FS	ZF	ZW
TGs (mmol/l)	Male	0.55 ± 0.27[a]	1.30 ± 0.52[b]	0.82 ± 0.38[a,b]	0.79 ± 0.65[a,b]
	Female	0.41 ± 0.27[a]	1.10 ± 0.69[b]	0.96 ± 0.57[a,b]	0.43 ± 0.35[a]
TC (mmol/l)	Male	5.40 ± 1.60[a]	8.80 ± 2.40[b]	5.60 ± 1.80[a]	6.10 ± 2.90[a,b]
	Female	8.10 ± 2.40[a,*]	11.00 ± 2.50[b,*]	7.60 ± 1.70[a,*]	8.20 ± 1.90[a,b,*]
HDL-c (mmol/l)	Male	1.70 ± 0.06[a]	1.70 ± 0.04[a]	1.70 ± 0.05[a]	1.70 ± 0.04[a]
	Female	1.70 ± 0.04[a,b]	1.70 ± 0.05[a,b]	1.60 ± 0.04[a,b]	1.70 ± 0.03[a,b]
LDL-c (mmol/l)	Male	3.70 ± 0.75[a]	5.00 ± 0.26[b]	3.60 ± 0.71[a]	4.00 ± 0.59[a]
	Female	4.80 ± 0.48[a,*]	6.50 ± 1.30[b,*]	4.30 ± 0.43[a,*]	4.90 ± 0.52[a,b,*]
TGs : HDL-c ratio	Male	0.32 ± 0.16[a]	0.75 ± 0.30[b]	0.48 ± 0.23[a,b]	0.47 ± 0.38[a,b]
	Female	0.24 ± 0.16[a]	0.61 ± 0.40[b]	0.55 ± 0.33[a,b]	0.25 ± 0.21[a]

Data expressed as mean ± standard deviation. [a,b] = within row means with different letters significantly different at $P < 0.05$; * = female rats had significantly higher level of cholesterol subtypes at $P < 0.0001$ compared to males; W = gavaged with 10 ml/kg body weight (bwt) of distilled water before weaning and then provided unlimited access to plain tap water post-weaning (M = 9, F = 11); FS = gavaged with 10 ml/kg bwt of a 20% fructose solution before weaning and then provided unlimited access to a 20% fructose solution to drink post-weaning (M = 9, F = 11); ZF = gavaged with a combination of 20% fructose solution (10 ml/kg bwt) and zingerone (40 mg/kg bwt) before weaning and then unlimited access to 20% fructose solution to drink post-weaning (M = 9, F = 12); ZW = gavaged with zingerone only at 40 mg/kg bwt dissolved in distilled water before weaning and given unlimited access to plain tap water to drink post-weaning (M = 8, F = 10); TGs = triglycerides; TC = total cholesterol; HDL c = high-density lipoprotein cholesterol; LDL c = low-density lipoprotein cholesterol.

Table 2. Effects of neonatally administered zingerone on glycaemic parameters of high-fructose fed rats in adulthood

Parameter	Sex	W	FS	ZF	ZW
Glucose (mmol/l)	Male	4.10 ± 0.38	3.80 ± 0.47	3.70 ± 0.18	4.10 ± 0.25
	Female	3.80 ± 0.47	3.70 ± 0.47	3.60 ± 0.42	3.90 ± 0.34
Insulin (ng/ml)	Male	0.85 ± 0.70	1.40 ± 0.58	1.20 ± 0.48	1.20 ± 0.63
	Female	1.00 ± 0.61	1.10 ± 0.46	1.20 ± 0.43	0.93 ± 0.62
HOMA-IR index	Male	0.15 ± 0.12	0.24 ± 0.12	0.20 ± 0.08	0.22 ± 0.11
	Female	0.17 ± 0.12	0.18 ± 0.09	0.19 ± 0.06	0.16 ± 0.11
Adiponectin (pg/ml)	Male	176.00 ± 99.00	275.00 ± 107.00	293.00 ± 221.00	253.00 ± 179.00
	Female	243.00 ± 106.00	228.00 ± 151.00	256.00 ± 140.00	232.00 ± 119.00

Data expressed as mean ± standard deviation. $P > 0.05$; W = gavaged with 10 ml/kg body weight (bwt) of distilled water before weaning and then provided unlimited access to plain tap water post-weaning (M = 9, F = 11); FS = gavaged with 10 ml/kg bwt of a 20% fructose solution before weaning and then provided unlimited access to a 20% fructose solution to drink post-weaning (M = 9, F = 11); ZF = gavaged with 10 ml/kg bwt of a 20% fructose solution combined with zingerone at 40 mg/kg bwt before weaning and then unlimited access to 20% fructose solution to drink post-weaning (M = 9, F = 12); ZW = gavaged with zingerone only at 40 mg/kg bwt dissolved in distilled water before weaning and given unlimited access to plain tap water to drink post-weaning (M = 8, F = 10); HOMA-IR = homeostatic model assessment of insulin resistance.

of HDL-c [Table 1; $P = 0.2847$ (males); $P = 0.3765$ (females)], fasting glucose [$P = 0.1042$ (males); $P = 0.3679$ (females)], insulin [$P = 0.3387$ (males); $P = 0.6824$ (females)] and adiponectin [$P = 0.4289$ (males); $P = 0.9563$ (females)], as well as the values of HOMA-IR [$P = 0.3033$ (males); $P = 0.9208$ (females)] were not different across the different treatment groups (Table 2) in both sexes.

Females rats had significantly higher levels of total [$P < 0.0001$ (sex and treatment effects), $P = 0.9590$ (interaction effect)], LDL [$P < 0.0001$ (sex and treatment effects), $P = 0.3259$ (interaction effect)] and HDL [$P = 0.0124$ (sex effect), $P = 0.0004$ (treatment and interaction effects)] cholesterols than their corresponding males. However, no sex difference was observed in the levels of TGs [$P = 0.2117$ (sex effect), $P < 0.0001$ (treatment effect), $P = 0.4545$ (interaction effect)] TGs to HDL-c ratio [$P = 0.1628$ (sex effect), $P = 0.0002$ (treatment effect), $P = 0.4530$ (interaction effect)] and glycaemic parameters ($P > 0.05$ for sex, treatment and interaction effects).

Visceral obesity

The visceral fat percentages of both male and female rats are shown in Fig. 2. In both sexes, high dietary fructose (FS; ZF) significantly [$P = 0.0037$ (males); $P < 0.0001$ (females)] increased visceral fat (percentage body mass) compared to the control (W; Fig. 2a and 2b). However, the zingerone only treated group (ZW) had similar relative visceral fat percentage with the controls in both sexes [$P = 0.1590$ (males); $P = 0.3414$ (females)].

Female rats significantly [$P < 0.0001$ (sex and treatment effects), $P = 0.3573$ (interaction effect)] had higher visceral fat percentage than males (Fig. 2c).

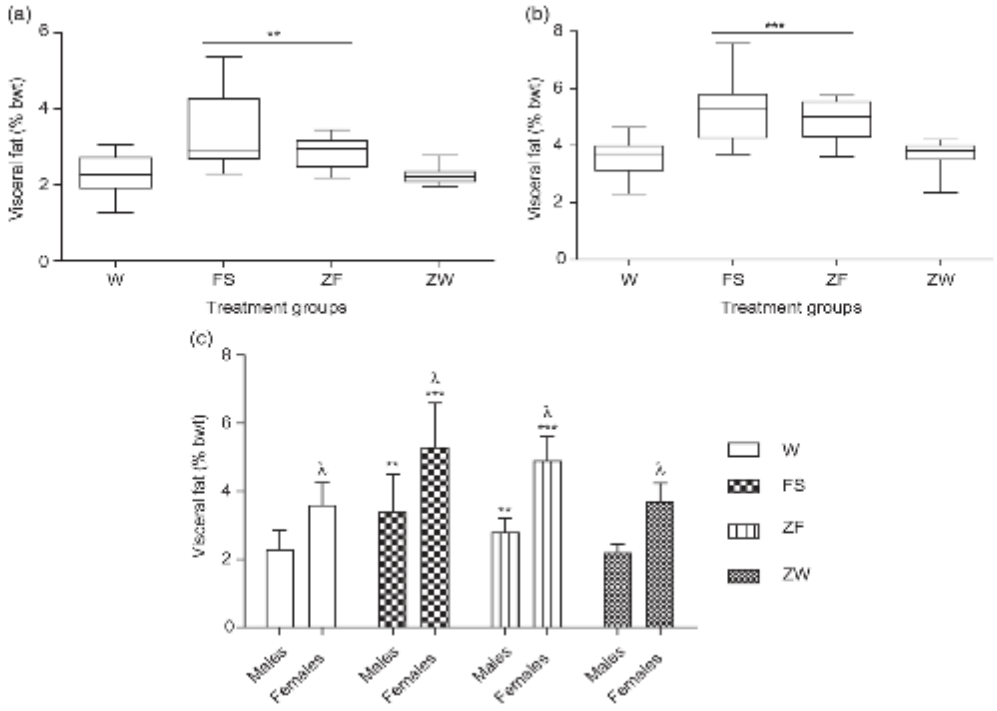

Fig. 2. Effects of neonatally administered zingerone on the relative mass of visceral fat percentage (% bwt) of high-fructose fed male (a) and female (b) rats in adulthood; and visceral fat comparism between males and females (c). Data expressed as mean ± standard deviation. ** = fructose treated groups in male rats (FS; ZF) had significantly higher visceral fat percentage at $P = 0.0037$ when compared to other treatments; *** = fructose treated groups in female rats (FS; ZF) had significantly higher visceral fat percentage at $P < 0.0001$ when compared to other treatments; λ = female rats had significantly higher visceral fat percentage at $P < 0.0001$ compared to males; W = gavaged with 10 ml/kg body weight (bwt) of distilled water before weaning and then provided unlimited access to plain tap water post-weaning (M = 9, F = 11); FS = gavaged with 10 ml/kg bwt of a 20% fructose solution before weaning and then provided unlimited access to a 20% fructose solution to drink post-weaning (M = 9, F = 11); ZF = gavaged with a combination of 20% fructose solution (10 ml/kg bwt) and zingerone (40 mg/kg bwt) before weaning and then unlimited access 20% fructose solution to drink post-weaning (M = 9, F = 12); ZW = gavaged with zingerone only at 40 mg/kg bwt dissolved in distilled water before weaning and given unlimited access to plain tap water to drink post-weaning (M = 8, F = 10); % bwt= percentage body weight.

Discussion

The study evaluated whether orally administered zingerone in the neonatal period would protect against the development later in adulthood, of long-term dietary fructose-induced metabolic derangements in Sprague-Dawley rats. In both sexes, it was observed that as adults, there were differences in fluid consumption such that rats provided fructose solution drank more fluid and had a greater total calorie intake than those that had plain water. Additionally, the rats provided fructose solution as drinking fluid ate less food (standard rat chow) than those provided plain water. Rats provided fructose solution to drink had increased terminal body masses (females), visceral adiposity and elevated plasma triglyceride and cholesterol concentrations at termination (both sexes). Administration of zingerone to neonatal rats programmed protection against high-fructose diet-induced increase in body mass (females) and hypercholesterolemia in adulthood (both sexes). Dimorphic responses were also observed with the manifestation of metabolic parameters.

The amount of food or water that is consumed by an organism depends on several intrinsic and extrinsic factors, including palatability, the aroma of the food and state of satiety.[13] The current study reports that the rats that received fructose solution consumed larger amounts of fluid (FS), but lesser amounts of rat chow when compared with those that did not receive fructose solution as drinking fluid in adult life in both sexes (Supplementary Tables S1A and S1B). The increase in fluid intake observed in the high-fructose diet-fed rats could have been due to the sweet taste of the fructose solution.[10,29] However, rats have intrinsic mechanisms that regulate calorie intake.[13] This could explain why there was a reduction in the consumption of solid food by the rats drinking fructose in order to maintain their calorie intake, preferentially from consumption of the fructose solution. In addition, 5 ml of 20% fructose solution (which contains 1 g fructose) yields more calories of energy than the 1 g of standard rat food.[29] Therefore, despite the food consumption being higher in the rats that received water (W and ZW), the rats that received fructose had a relatively higher caloric intake from fructose than the other rats (Supplementary Table S1C).

It was observed in this study that female rats consumed relatively more food and fluid (and thus had more calories) than males in adulthood. Dimorphic responses have been reported on rats' feeding, with females having higher intake than males.[33] The increase in food intake observed in females could be due to increase in ghrelin levels that is reported to be higher in females.[33] However, we did not assay ghrelin levels in this study. In addition, females have a been reported to have a greater preference for sweet solutions than males.[34] This could probably be due to oestrogen effects on the satiety centre.[35] An increase in fructose consumption could

lead to metabolic complications including increasing the body mass of the rats.

The induction body mass of the rats was not different across the treatments in both sexes. This reflects the deliberate choice to use rats born in litter sizes of 8–12, thereby avoiding the impact of litter size on induction mass which could impact on the study outcomes, as small litter sizes are prone to faster growth and obesity compared to large litter sizes.[36] The suckling period is characterised by rapid growth which can be altered (positively or negatively) by dietary interventions.[37] The high fructose diet and/or zingerone had no effect on the weaning (PND 21) body mass of the male and female pups in this study, suggesting that both fructose and zingerone did not negatively affect growth during the neonatal period. Although the terminal body mass of the male rats was not affected by the interventions, the high-fructose diet increased the terminal body mass of the female rats compared to the other rats. Zingerone programmed protection against the high fructose diet-induced increase in body mass in female rats. Fructose is known to increase body mass by increasing hepatic *de novo* lipogenesis and tissue's adipogenesis that contribute to the development of obesity.[11] Thus, it could be speculated that neonatal orally administered zingerone prevented the fructose-induced increase in terminal body masses by programming protection (via regulating hepatic fatty acid oxidation) against fructose-induced hepatic lipogenesis and tissue's adipogenesis in the female rats.[38] The higher terminal body mass observed in males in comparison to females could be due to the greater trophic effects of testosterone and growth hormone in males compared to females.[39,40] An increase in body mass is associated with visceral obesity that is known to result in poor cardio-metabolic health.[41]

The present study found that a long-term high-fructose diet had programmed for increased visceral adiposity in both male and female rats. The high-fructose diet-fed rats that were administered neonatal zingerone had a 26% (males) and 11% (females) reduction in mean visceral fat pad mass compared to those who were fed the high-fructose diet alone. This means that neonatal zingerone had programmed for a reduction in the visceral adiposity observed with fructose fed rats. It has been reported that complete attenuation of obesity is not usually achievable in practical situations; however, a 5–10% weight loss significantly decreases the risk of morbidity and mortality.[42] Although not significantly different statistically, an 11% reduction in the visceral fat showed that neonatal zingerone could potentially attenuate the risk of developing cardio-metabolic complications later in life, since increased visceral obesity has been linked to the development of cardio-metabolic disorders including dyslipidaemia.[41]

The higher visceral fat percentage observed in female rats than males could be explained by the fact that females have a greater tendency of fat accumulation than males, an effect that has been ascribed to female sex hormones.[42] This finding had reinforced that of terminal body masses observed in both sexes, in which fructose had increased the terminal body mass in females but not in males. An increase in adiposity is invariably associated with dyslipidaemia.[41]

Indeed, in the current study, the long-term intake of a high-fructose diet resulted in hypertriglyceridaemia, hypercholesterolemia, hyper-LDL-cholesterolemia in both sexes. Dyslipidaemia is a key component of metabolic syndrome that can lead to cardiovascular diseases including hypertension and atherosclerosis.[12] The triglyceride to HDL-cholesterol ratio is an atherogenic index that is used to assess the risk of developing metabolic syndrome and coronary artery disease.[44] This study found that long-term intake of a high-fructose diet increased the triglycerides to HDL-cholesterol ratio in adulthood suggesting a greater risk of developing metabolic syndrome and related cardiovascular diseases.[45] Neonatal zingerone prevented the fructose-induced dyslipidaemia, thus zingerone had programmed protection against fructose-induced dyslipidaemia in adulthood. These anti-hyperlipidaemic effects of zingerone have been observed in streptozotocin-induced diabetic adult rats.[23] It was reported that zingerone exerts its anti-hyperlipidaemic effects by stimulating lipolysis and enhancing the activity of norepinephrine-sensitive lipases.[26] We observed sex differences in the levels of cholesterol subtypes such that cholesterol was elevated in female rats in this study. This is not surprising since females are more prone to dyslipidaemia than males, which could have been due to oestrogen effect.[43] Fructose-induced dyslipidaemia can result without affecting glycaemic parameters in rats.[47]

Despite fructose consumption having been implicated in the aetiology of hyperglycaemia, hyperinsulinaemia, insulin resistance and hypoadiponectinaemia in adult rats,[48] these parameters were not affected by the interventions of the current study, in both sexes. This indicated that administration of 20% fructose solution did not programme for the development of hyperglycaemia, hyperinsulinaemia, insulin resistance and low plasma adiponectin in adulthood. These findings contradict that of Ibitoye and Ajiboye[48] who reported dysglycaemia and hypoadiponectinaemia following administration of a high fructose diet to adult Wistar rats for 6 weeks. The variance could be due to the rats' age at commencement of the study, strain and the mode of fructose administration. High-fructose diets where fructose is mixed into feed tend to produce features of metabolic dysfuction more readily than fructose intake as a drinking solution;[49] likewise, Wistar rats are more prone to manifest features of metabolic syndrome than Sprague-Dawley rats.[29] Older rats are also more susceptible to the effects of fructose than younger rats due to innate protective mechanisms present at young age.[49] As consumption of fructose did not affect the glycaemic parameters, the potential protective programming effect of neonatal zingerone against dysglycaemia and insulin resistance in adulthood could not apparently manifest, and this needs future investigation.

Our study is novel in showing that zingerone administered in the neonatal phase can programme long-term protection against high-fructose diet-induced dyslipidaemia. The precise mechanisms need to be further explored; however, it is likely that zingerone could have dysregulated the proteins and receptors involved in lipid metabolism such as PPARα thereby regulating fatty acid oxidation and preventing the fructose-induced lipogenesis and dyslipidaemia.[25] As mentioned in the introduction section, the liver also plays a central role in lipid metabolism, and the impact of the interventions on the liver needs to be investigated in future studies.

Conclusion

This study has demonstrated that long-term fructose intake caused visceral obesity and dyslipidaemia in both male and female rats. The novelty of our study is that oral administration of zingerone to rats during the neonatal period programmed for long-term protection against the development of high-fructose diet-induced visceral obesity and dyslipidaemia later in adult life. Thus, a similar strategic use of zingerone neonatally could be explored in humans to manage diet-induced metabolic syndrome, thereby replacing

the use of animal models in exploring the therapeutic effects of zingerone.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S2040174420000525

Acknowledgements. Staff of the Central Animal Service Unit, Faculty of Health Sciences, University of the Witwatersrand are hereby acknowledged for assistance with the care of the animals. Gratitude is extended to Miss Monica Gomes for her technical assistance with the metabolic assays. Dr Trevor Nyakudya, Ms Thobekile Precious Dladla and Mr Bayanda Mdoda are also acknowledged for their assistance during the terminal procedures.

Financial Support. The authors acknowledge the Medical Faculty Research Endowment Fund, Faculty of Health Sciences Research Committee and School of Physiology of the University of Witwatersrand for providing the research funds (Grant Number: 001 254 8521101 5121105 000000 0000000000 5254). The National Research Foundation (NRF) Thuthuka Fund (Grant Number: TTK170415227205) is acknowledged for their financial support. The Federal University Birnin Kebbi (Nigeria) and Tertiary Education Trust Fund of Nigeria are also acknowledged for providing funds required for feeding, accommodation and transportation during the conduct of the study.

Conflicts of Interest. None declared.

Ethical standards. We confirm that the study was conducted according to the internationally accepted principles for laboratory animal use and care as stipulated in South African National Standard (SANS 10386:2008) and Animals Protection Act, 1962: Act No. 71 and was approved by the Animal Research Ethics Committee (AREC) of the University of Witwatersrand (Reference No: 2017/010/71/B).

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J Med Food 00 (0) 2021, 1–9
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DOI: 10.1089/jmf.2020.0189

Zingerone Administered Neonatally Prevents the Subsequent Development of High Dietary Fructose-Induced Fatty Liver in Sprague Dawley Rats

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ABSTRACT Consumption of high-fructose diets early in life increases the risk of developing metabolic disorders, including nonalcoholic fatty liver disease (NAFLD). Zingerone, an alkaloid isolated from *Zingiber officinale*, has been demonstrated to reverse obesity and fatty liver in adult male rats. We investigated the potential preventive effects of neonatally administered zingerone on the development of fructose-induced NAFLD in male and female rats. Four-day-old male ($n = 35$) and female ($n = 44$) rat pups were randomized and gavaged with: 10 mL/kg body weight (bwt) of distilled water (C), 10 mL/kg bwt of 20% fructose solution (Fr), 10 mL/kg bwt of 20% fructose solution +40 mg/kg bwt of zingerone (ZFr), and 40 mg/kg bwt of zingerone (Z) daily for 14 days. After weaning, all groups continued on unlimited standard rat feed; however, groups C and Z had plain drinking water, whereas groups Fr and ZFr had unlimited 20% fructose solution to drink for 10 weeks. Rats on the high-fructose diet (Fr) compared with the negative controls (C) had significantly increased hepatic lipid content (in %, males: $P = .0002$; females: $P < .0001$, analysis of variance [ANOVA]) and hepatic steatosis score (in %, males: $P = .0018$; females: $P < .0022$, Kruskal–Wallis ANOVA). Zingerone prevented ($P < .05$) the fructose-induced increase in hepatic steatosis in both sexes. The plasma alanine aminotransferase activity, levels of uric acid, TBARS (thiobarbituric acid reactive substances), IL-6 (interleukin-6), and TNF-α (tumor necrosis factor alpha) were not different ($P > .05$, ANOVA) across the different treatment groups in both sexes. No difference ($P > .05$, ANOVA) was observed between the two sexes for treatment, sex and interaction effects with regard to hepatic lipid content, and measured blood parameters. The use of zingerone neonatally should be further investigated as a strategic prophylactic intervention for the prevention of long-term high-fructose diet-induced NAFLD.

KEYWORDS: • *early postnatal period* • *NAFLD* • *Sprague Dawley* • *sugary diets* • *vanillyl acetone*

INTRODUCTION

THE AVERAGE GLOBAL prevalence of nonalcoholic fatty liver disease (NAFLD) is 25%.[1] This prevalence increases with increasing age and male sex.[2] NAFLD is a clinical condition characterized by fatty infiltration of hepatocytes in the absence of significant alcohol consumption.[3] Histologically, NAFLD initially presents as simple steatosis, which in 10–15% of patients, progresses to nonalcoholic steatohepatitis (NASH).[3] NASH is characterized by inflammation and can further progress to fibrosis and eventually cirrhosis of the liver leading to hepatocellular carcinoma.[4] NAFLD is asymptomatic in most patients but associated with obesity and risk factors of metabolic syndrome.[5] It is the most common cause of chronic liver disease globally.[5] Despite limited data on the global economic burden of managing NAFLD, the annual medical cost of managing NAFLD is ~62 billion dollars in the United States,[6] and the cost of liver transplants is expected to rise with increasing prevalence of the condition.[7]

Despite NAFLD being associated with the components of metabolic syndrome (obesity, dyslipidemia, or insulin resistance),[8] recent evidence shows that NAFLD can develop independently in the absence of the aforementioned metabolic disorders.[9] Nutrition, environment, gut microbiota, and genetics/epigenetics are among the factors implicated in the pathogenesis of NAFLD.[4]

Environmental and nutritional influences during periods of developmental plasticity, such as the neonatal period, can result in epigenetic changes, which can alter phenotype physiology and susceptibility to the development of metabolic diseases, including NAFLD, later in life.[10] Excessive intake of fructose during the perinatal period may serve to sensitize the liver to subsequent nutritional, metabolic, and environmental events, which trigger the development of NAFLD, hepatomegaly, and increase in general body mass later in life.[4,11,12] Additionally, high dietary fructose consumption is a major cause of NAFLD due to the unregulated nature of fructose metabolism.[13]

Prolonged excessive hepatic fructose metabolism causes adenosine triphosphate (ATP) depletion, which stimulates

Manuscript received 7 October 2020. Revision accepted 16 December 2020.

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uric acid synthesis leading to mitochondrial dysfunction and oxidative stress.[13] These events stimulate the production of reactive oxygen species that eventually lead to inflammation characterized by elevated proinflammatory cytokines, apoptosis, and fibrosis.[4]

Metabolism of fructose also affects the expression of genes and receptors involved in the different steps of hepatic lipid accumulation and hepatic inflammation.[14] These genes and receptors include those associated with carbohydrate response element-binding protein (ChREBP), sterol regulatory element binding protein 1 (SREBP-1), acetyl-CoA carboxylase 1, carnitine palmitoyltransferase 1, tumor necrosis factor alpha (TNF-α), and peroxisome proliferator-activated receptor alpha (PPARα).[14] Dysregulation of these genes and receptors can lead to the development of NAFLD secondary to hepatic lipid accumulation and inflammation.[4]

The inflammatory, apoptotic, and lipid peroxidation processes associated with NAFLD can generate TNF-α, interleukin-6 (IL-6), and thiobarbituric acid reactive substances (TBARS),[5] which damage hepatocytes leading to elevation of key liver enzymes in circulation, particularly alanine aminotransferase (ALT), although this elevation does not correlate with the extent of steatosis, inflammation, or fibrosis.[5] NAFLD can be managed effectively in its early stages of pathological development.

Despite no specifically approved pharmacological drug treatment for NAFLD,[1] regular exercise and good dietary habits are the first-line approaches in the early management of NAFLD.[15] The use of synthetic pharmacological agents, such as fenofibrate used in the management of specific risk factors of metabolic syndrome, is limited as these agents are usually expensive, require chronic use, inaccessible in some communities and monotherapeutic.[16,17] There is need for innovative prophylactic interventions against NAFLD using locally available resources.

Globally, communities are increasingly opting for plant-derived medications for their health care needs.[16] The medicinal potential of natural agents for protection against adverse neonatal metabolic programming of NAFLD needs to be explored. Previous studies have used fructose-fed rats during the immediate postnatal period to program for long-term metabolic health outcomes using phytochemicals. Neonatal administration of ursolic acid,[18] oleanolic acid,[19] and *S*-allyl cysteine[20] prevented the development of hepatic lipid accumulation in rats fed high-fructose diets. In the current study, we investigated whether zingerone could be used for that purpose.

Zingerone (vanillyl acetone) is an alkaloid found in the rhizomes of the plant ginger (*Zingiber officinale*). Studies have documented the hepatoprotective and antiobesity properties of zingerone in adult rat and mouse experimental models of metabolic disorders.[21–24] The mechanisms by which zingerone exerts its hepatoprotective effects include its ability to upregulate lipolysis, scavenge free radicals, and downregulate inflammatory cytokines in the liver.[21,24]

Given the heavy burden that management of NAFLD places on health care systems globally and the absence of specific treatments for NAFLD, there is need for strategic prophylactic interventions. The biological properties of zingerone made it a suitable candidate phytochemical in the current study for administration during the neonatal period to explore its potential to protect against long-term development of NAFLD secondary to high-fructose diets.

MATERIALS AND METHODS

Experimental setting and procedures

The study was approved by the Animal Research Ethics Committee (AREC) of the University of the Witwatersrand (Clearance No: 2017/010/71/B) and conducted at the Central Animal Service (CAS) of the University of the Witwatersrand, Johannesburg, South Africa, using internationally accepted principles for laboratory animal use and care (South African National Standard [SANS 10386:2008] and Animals Protection Act, 1962: Act No. 71). The Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines were adopted while compiling the article.

The rats used in this study were obtained from the CAS, University of the Witwatersrand. Seven nulliparous dams of Sprague Dawley rats (*Rattus norvegicus*), each with a litter consisting of 8–12 four-day-old suckling male and female pups weighing 9.7 ± 1.1 g, were used in this study. The dams with their respective litters were housed in Perspex cages containing wood shavings as bedding. The room temperature was maintained at 25°C ± 1°C and a 12-h light and dark cycle with lights off from 7 pm to 7 am. The dams were supplied with standard rat chow (LabChef Rodent Breeder [Pty] Ltd., Stellenbosch, South Africa) and *ad libitum* water access throughout the suckling period. The pups were allowed to freely nurse from their respective dams until weaning on postnatal day 21. The dams were returned to stock after weaning of their pups. The weaned rats were then housed individually in Perspex cages having the same environmental conditions described earlier.

The study consisted of two main stages (Fig. 1): a preweaning stage (postnatal day 8–21) preceded by a 4-day adaptation period and a postweaning stage (postnatal day 22–91). On postnatal day 8, the pups ($n = 79$; 35 males, 44 females) from each litter were randomly allocated to four treatment groups: Group 1: received 10 mL/kg body weight (bwt) of distilled water gavaged orally before weaning (daily) and *ad libitum* access to plain drinking tap water after weaning (9 males, 11 females); Group 2: received 10 mL/kg bwt of a 20% fructose solution gavaged orally before weaning (daily) and *ad libitum* access to a 20% drinking fructose solution after weaning (9 males, 11 females) to induce metabolic dysfunction; Group 3: in which we investigated whether neonatal zingerone could program for protection against long-term high-fructose consumption, received 10 mL/kg bwt of a 20% fructose solution + zingerone at 40 mg/kg bwt gavaged orally before weaning (daily) and *ad libitum* access to a 20% drinking fructose solution after weaning (9 males, 12 females); Group 4: received 40 mg/kg bwt of zingerone dissolved in 10 mL/kg bwt of distilled water gavaged orally before weaning (daily) and *ad libitum*

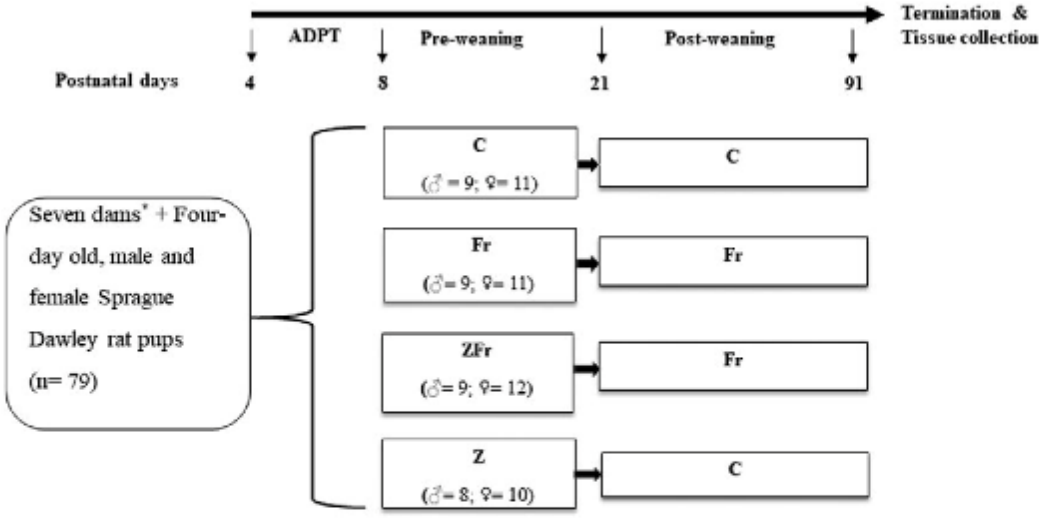

FIG. 1. Flow diagram showing the timeline and treatment groups. *Only with pups during the adaptation and preweaning phases: C, plain water; FR, 20% fructose solution; ZFr, zingerone (40 mg/kg bwt) +20% fructose solution; Z, zingerone (40 mg/kg bwt); ADPT, adaptation; ♂, males; ♀, females.

access to plain drinking tap water after weaning (8 males, 10 females) to assess the effects of zingerone alone on metabolic health of the animals.

Fructose solution at 20% has been shown to induce metabolic dysfunction in rats,[18] whereas zingerone at 40 mg/kg bwt has been shown to prevent against ethanol-induced hepatotoxicity in rats,[25] hence these doses were chosen for the current study.

The fructose solution (Natures Choice, South Africa) was constituted to 20% by adding 20 g of fructose into 100 mL of water,[26] whereas food-grade zingerone (Catalogue No. W312401-1KG) was purchased from Sigma–Aldrich.

The rats were weighed daily during the preweaning stage and then twice a week during the postweaning stage by individually placing the rats in a preweighed cage on an electronic balance (Snowrex Electronic Scale, Clover Scales, Johannesburg).

Procedures at termination

After the 12-week intervention period, the rats were fasted overnight, terminal body mass measured, and then euthanized with an appropriate and acceptable euthanasia in rats, an intraperitoneal injection of sodium pentobarbitone[27] (Centaur Laboratories, Johannesburg, South Africa) at 150 mg/kg bwt. After euthanasia, a ventral midline incision was made through the thorax and abdomen; syringes with attached hypodermic needles were used to withdraw blood samples via intracardiac access. The blood samples were then transferred into heparinized tubes (BD Vacutainer, Plymouth, United Kingdom) and centrifuged (Rotofix 32A; Hettich Zentrifugen, Germany) at 3700 *g* for 15 min at 20°C. The plasma was harvested and stored at −20°C for determination of ALT, uric acid, and TBARS. The liver was carefully dissected out from the abdominal cavity and weighed using a Precisa 310M balance (Precision Instruments, Johannesburg, South Africa). The right medial lobe of the liver was preserved in 10% phosphate-buffered formalin for histological analysis, while the remaining liver lobes were preserved at −20°C for quantification of lipid content.

Determination of hepatosomatic index

The terminal body mass and the fresh whole liver weight were used to compute the hepatosomatic index[28]:

$$\text{Hepatosomatic index (\%)} = [\text{mass of liver (g)}/\text{body mass (g)}] \times 100.$$

Determination of hepatic lipid content

The hepatic lipids were determined by solvent extraction using a Soxhlet apparatus (Gebr. Rettberg GmbH, Göttingen, Germany), according to the Official Methods of Analysis of the Association of Official Agricultural Chemists (AOAC) International, 2005, Method 960.39. Briefly, the liver samples were lyophilized in a freeze-dryer (VirTis BenchTop BK-FD12; SP Scientific, New York) and milled into a fine powder. The milled liver samples were then extracted using petroleum ether in a Soxhlet apparatus and the lipid quantified gravimetrically.

Histological assessment of the liver

The right medial lobes of the liver samples that were preserved in 10% phosphate-buffered formalin were processed using an automatic tissue processor (Microm STP 120; Thermo Scientific, MA), embedded in paraffin wax, sectioned at 5 μm, and then stained with hematoxylin and

eosin (H&E) for steatosis and inflammation assessment and Masson's trichrome (MT) for assessment of fibrosis. The slides were viewed under a light microscope (40×) (Leica Biosystems) mounted to a camera, which uses ZEISS ZEN microscope software for image capture, and scored for steatosis, collagen deposition, and fibrosis.

The H&E images were used to score micro- and macrovesicular steatoses as described by Kleiner *et al*.[29] The grading was performed based on the total area affected per camera field, according to the following criteria: Grade 0 steatosis: <5% fat droplets; Grade 1 steatosis: 5–33% fat droplets; Grade 2 steatosis: 34–66% fat droplets; and Grade 3 steatosis: >66% per camera field at 40× magnification. Inflammation was scored by counting the foci of inflammatory cells in the liver parenchyma per camera field and graded as follows: Grade 0 = no foci of inflammation; Grade 1 = less than 2 foci; Grade 2 = 2–4 foci; Grade 3 = greater than 4 foci.

Fibrosis was assessed from photomicrographs of the MT-stained sections at 40× magnification.

Determination of ALT, uric acid, TBARS, and inflammatory markers in plasma

Plasma ALT activity and uric acid concentration were determined using a calibrated VetTest analyzer (IDEXX VetTest® Clinical Chemistry Analyzer; IDEXX Laboratories, Inc.). TBARS, IL-6, and TNF-α were determined using rat-specific Enzyme-Linked Immunosorbent Assay kits (Elabscience Biotechnology Co., Ltd., Houston, TX), respectively.

Statistical analysis

Data were analyzed using GraphPad Prism 8 software (GraphPad Software, Inc., San Diego). Parametric data (hepatosomatic index, ALT, TBARS, IL-6, TNF-α, and hepatic lipid content) were analyzed by one-way analysis of variance (ANOVA) and expressed as mean ± standard deviation, followed by Tukey *post hoc* tests for comparison of the means. The Kruskal–Wallis test (nonparametric one-way ANOVA) was used to analyze multiple-group data for steatosis grades, inflammation, and fibrosis and expressed as median ± range, followed by Dunn's *post hoc* test to compare the medians. Sex variations were analyzed using two-way ANOVA. Statistical significance was considered when $P < .05$.

RESULTS

Hepatosomatic index and hepatic lipid accumulation

The mass of the liver relative to body weight was not different (males: $P = .6217$; females: $P = .0657$) across the different treatment groups in both sexes (Table 1). No sex difference was observed between male and female rats regarding percent liver weight (sex effect: $P = .9999$, treatment effect: $P = .4540$, and interaction effect: $P = .3500$).

Long-term fructose consumption significantly increased the hepatic lipid accumulation in both male ($P = .0002$) and female ($P < .0001$) rats compared with all other groups (Table 1). Thus, the neonatally administered zingerone prevented the fructose-induced hepatic lipid in both sexes, such that the zingerone-treated (with or without fructose) group had a similar (males: $P = .7632$; females: $P = .5103$) hepatic lipid content compared with negative controls. There was no sex difference observed (sex effect: $P = .3774$, treatment effect: $P < .0001$, and interaction effect: $P = .8416$) with regard to percent hepatic lipids accumulation.

Histomorphometry of the liver

Figure 2A and B shows representative photomicrographs of the liver histology from each of the experimental groups (H&E stain) of male and female rats, respectively. There was a notable fatty infiltration (macro- and microvesicular steatosis indicated by solid and dash arrows, respectively) in the sections of the fructose-only-treated group, which was generally absent in the other treatment groups (Fig. 2A [males]; Fig. 2B [females]). This was confirmed by the micro- and macrovesicular steatosis scores, which were greater in rats fed a high-fructose diet only compared with the other treatment groups in both sexes ($P < .05$, Dunn's multiple comparison; Table 2). This demonstrated that zingerone prevented the development of steatosis induced by high-fructose diet in the study rats. No difference was

Table 1. Effects of Neonatally Administered Zingerone on the Hepatosomatic Index and Hepatic Lipid Accumulation of High-Fructose Diet-Fed Rats

Parameter	*Sex*	*C*	*Fr*	*ZFr*	*Z*
Hepatosomatic index (%)	Male	3.00 ± 0.21	3.00 ± 0.67	3.10 ± 0.15	3.00 ± 0.14
	Female	2.90 ± 0.14	3.10 ± 0.21	3.10 ± 0.20	3.00 ± 0.19
Hepatic lipids (% liver mass)	Male	4.50 ± 1.60[a]	8.30 ± 2.20[b]	5.20 ± 1.60[a]	4.70 ± 1.70[a]
	Female	4.50 ± 1.60[a]	8.50 ± 2.40[b]	6.20 ± 1.90[a]	5.00 ± 1.60[a]

Data are expressed as mean ± standard deviation.

[ab]Row means with different letters (ab) significantly different at $P = .0002$ (males) and $P < .0001$ (females).

C, 10 mL/kg bwt of distilled water before weaning and free access to plain tap water after weaning ($n = 20$; 9 males, 11 females); Fr, 10 mL/kg bwt of a 20% fructose solution before weaning and free access to a 20% fructose solution after weaning ($n = 20$; 9 males, 11 females); ZFr, 10 mL/kg bwt of a 20% fructose solution combined with zingerone at 40 mg/kg bwt before weaning and free access to a 20% fructose solution after weaning ($n = 21$; 9 males, 12 females); Z, zingerone only at 40 mg/kg bwt in 10 mL/kg bwt of distilled water before weaning and free access to plain tap water after weaning ($n = 18$; 8 males, 10 females).

bwt, body weight.

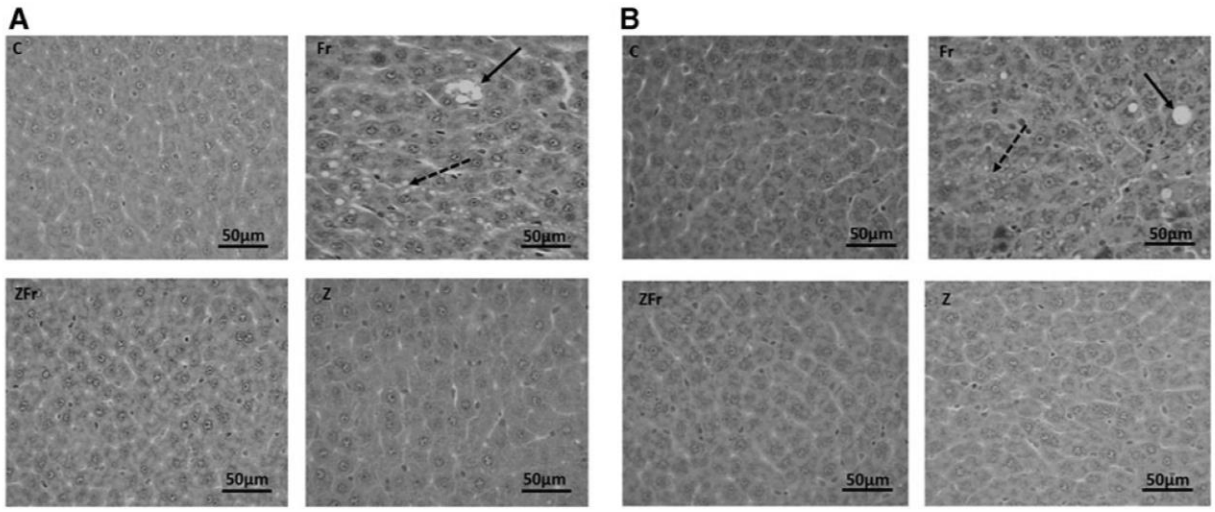

FIG. 2. Representative photomicrographs of the male **(A)** and female **(B)** rat liver sections stained with hematoxylin and eosin (40×). C, 10 mL/kg bwt of distilled water before weaning and free access to plain tap water after weaning ($n=20$; 9 males, 11 females); Fr, 10 mL/kg bwt of a 20% fructose solution before weaning and free access to a 20% fructose solution after weaning ($n=20$; 9 males, 11 females); ZFr, 10 mL/kg bwt of a 20% fructose solution combined with zingerone at 40 mg/kg bwt before weaning and free access to a 20% fructose solution after weaning ($n=21$; 9 males, 12 females); Z, zingerone only at 40 mg/kg bwt in 10 mL/kg bwt of distilled water before weaning and free access to plain tap water after weaning ($n=18$; 8 males, 10 females); the *dash arrows* point to microsteatosis; *solid arrows* point to macrosteatosis; scale bar = 50 μm. bwt, body weight.

observed between males and females on the steatosis score for sex ($P=.1889$), treatment ($P=.0826$), and interaction ($P=.7528$) effects. Additionally, there were no observable inflammatory cell aggregates in both sexes.

For the MT histological sections (MT stain), there was no observable fibrosis across the different treatment groups in both sexes (Fig. 3A [males]; Fig. 3B [females]).

Plasma ALT activity and concentrations of uric acid, TBARS, and inflammatory markers

There were no differences in the activity of ALT (males: $P=.0633$; females: $P=.2832$), concentrations of uric acid (males: $P=.5159$; females: $P=.7666$), TBARS (males: $P=.9889$; females: $P=.2282$), IL-6 (males: $P=.8979$; females: $P=.9867$), and TNF-α (males: $P=.3966$; females: $P=.4457$) across all the treatment groups (Table 3). Males had higher ALT activity than females (sex effect: $P<.0001$, treatment effect: $P=.0114$, and interaction effect: $P=.0626$); otherwise no sex difference ($P>.05$) was observed in the levels of uric acid, TBARS, IL-6, and TNF-α for sex, treatment, and interaction effects.

TABLE 2. EFFECTS OF NEONATALLY ADMINISTERED ZINGERONE ON THE STEATOSIS SCORES OF HIGH-FRUCTOSE DIET-FED RATS

Parameter	*Sex*	*C*	*Fr*	*ZFr*	*Z*
Microsteatosis	Male	0 (0–0)[a]	1 (0–3)[b]	0 (0–0)[a]	0 (0–0)[a]
	Female	0 (0–2)[a]	2 (0–3)[b]	0 (0–1)[a]	0 (0–2)[a]
Macrosteatosis	Male	0 (0–1)[a]	1 (0–2)[b]	0 (0–1)[a]	0 (0–1)[a]
	Female	0 (0–0)[a]	1 (0–2)[b]	0 (0–0)[a]	0 (0–1)[a]

Data are expressed as median (interquartile range).

[ab]Row means with different letters (ab) significantly different at $P<.05$.

C, 10 mL/kg bwt of distilled water before weaning and free access to plain tap water after weaning ($n=20$; 9 males, 11 females); Fr, 10 mL/kg bwt of a 20% fructose solution before weaning and free access to a 20% fructose solution after weaning ($n=20$; 9 males, 11 females); ZFr, 10 mL/kg bwt of a 20% fructose solution combined with zingerone at 40 mg/kg bwt before weaning and free access to a 20% fructose solution after weaning ($n=21$; 9 males, 12 females); Z, zingerone only at 40 mg/kg bwt in 10 mL/kg bwt of distilled water before weaning and free access to plain tap water after weaning ($n=18$; 8 males, 10 females).

DISCUSSION

The present study investigated the potential protective effects of neonatal administration of oral zingerone on long-term dietary fructose-induced fatty liver in Sprague Dawley rats. Hepatic lipid accumulation and fatty infiltration of hepatocytes were observed when the male and female rats were terminated in early adulthood, following long-term administration of the 20% fructose solution. Zingerone administered orally to the neonatal rats protected them against high-fructose diet-induced increased hepatic lipid accretion and steatosis (both sexes). Thus, neonatal administration of oral zingerone was effective in attenuating the development of long-term fructose-induced NAFLD in both male and female rats. A sexually dimorphic response was observed only in plasma ALT activity where male rats had more activity of the enzyme than their female counterparts.

The mass of the liver relative to body mass is known as the hepatosomatic index,[30] and it can be affected by nutritional status[31] and hepatic pathology including fatty liver diseases.[30] An increase in hepatosomatic index can result from a severe form of fatty liver disease including

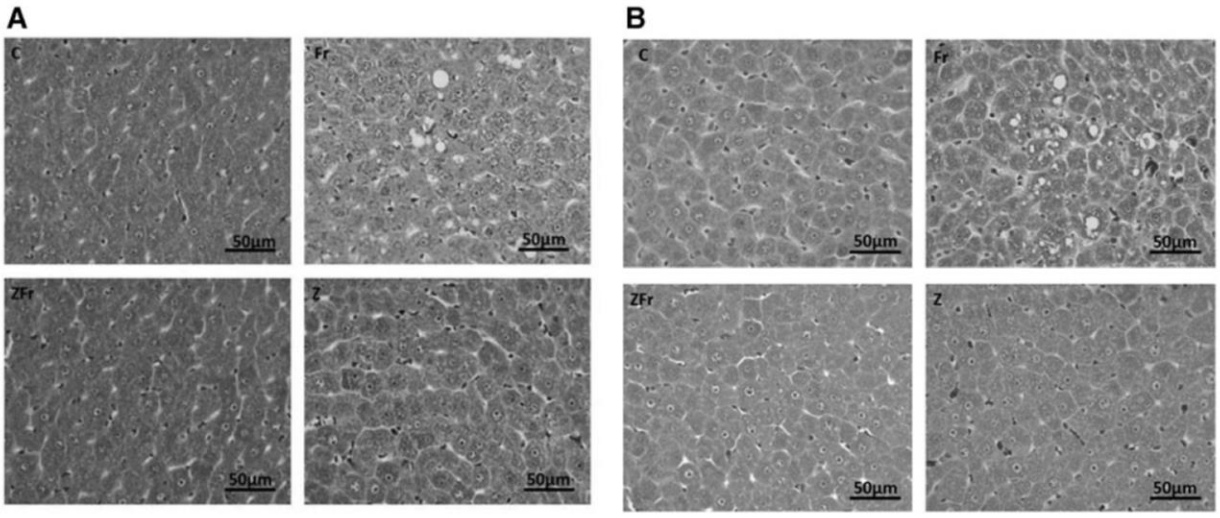

FIG. 3. Representative photomicrographs of the male **(A)** and female **(B)** rat liver sections stained with Masson's trichrome (40×). C, 10 mL/kg bwt of distilled water before weaning and free access to plain tap water after weaning ($n=20$; 9 males, 11 females); Fr, 10 mL/kg bwt of a 20% fructose solution before weaning and free access to a 20% fructose solution after weaning ($n=20$; 9 males, 11 females); ZFr, 10 mL/kg bwt of a 20% fructose solution combined with zingerone at 40 mg/kg bwt before weaning and free access to a 20% fructose solution after weaning ($n=21$; 9 males, 12 females); Z, zingerone only at 40 mg/kg bwt in 10 mL/kg bwt of distilled water before weaning and free access to plain tap water after weaning ($n=18$; 8 males, 10 females); there was no observable inflammation or fibrosis; scale bar = 50 μm.

NASH,[30] whereas a decrease in hepatosomatic index can result from undernutrition.[31] In the current study, we observed an increase in intrahepatic lipid accumulation in the high-fructose-fed male and female rats, without affecting the hepatosomatic index. This finding is in agreement with Mamikutty *et al.*[12] who reported increased hepatic lipid deposition without an increase in liver weight in adult male Wistar rats that consumed 20% fructose solution for 8 weeks. The hepatosomatic index that was not affected could be that the amount of lipid vacuoles in the hepatocytes of the study rats had not reached the threshold for inducing changes in the liver weight.[12] This finding indicated that the observed steatosis was not severe enough to cause NASH that is usually associated with increase in hepatosomatic index.[30]

However, the neonatally administered zingerone conferred protection against the long-term dietary fructose-induced hepatic lipids accretion in both sexes. A previous study also reported the hepatoprotective effect of zingerone in adult male rats, in which zingerone was administered orally at 100 mg/kg bwt for 8 weeks, and it prevented the intrahepatic lipid accumulation.[24] We have shown that by

TABLE 3. EFFECTS OF NEONATALLY ADMINISTERED ZINGERONE ON THE CONCENTRATIONS OF ALANINE AMINOTRANSFERASE, URIC ACID, THIOBARBITURIC ACID REACTIVE SUBSTANCES, AND LEVELS OF INFLAMMATORY MARKERS OF HIGH-FRUCTOSE DIET-FED RATS

Parameter	*Sex*	*C*	*Fr*	*ZFr*	*Z*
ALT (U/L)	Male	75.00 ± 16.00[a]	66.00 ± 17.00[a]	53.00 ± 8.80[a]	81.00 ± 36.00[a]
	Female	53.00 ± 8.70	47.00 ± 8.00	50.00 ± 6.80	52.00 ± 7.90
Uric acid (mg/dL)	Male	0.99 ± 0.56	1.30 ± 0.69	0.88 ± 0.42	1.10 ± 0.81
	Female	0.76 ± 0.61	1.00 ± 0.55	0.92 ± 0.42	0.89 ± 0.66
TBARS (μM)	Male	22.00 ± 3.50	23.00 ± 3.80	22.00 ± 4.40	22.00 ± 3.60
	Female	22.00 ± 4.10	21.00 ± 4.30	24.00 ± 3.80	20.00 ± 4.60
IL-6 (pg/mL)	Male	60.74 ± 23.17	66.98 ± 17.72	60.57 ± 21.14	64.62 ± 21.93
	Female	65.26 ± 18.97	66.12 ± 33.47	62.52 ± 19.16	64.97 ± 24.37
TNF-α (pg/mL)	Male	125.99 ± 19.97	152.02 ± 42.08	140.76 ± 21.30	147.97 ± 45.44
	Female	132.66 ± 50.00	143.85 ± 45.48	163.80 ± 54.31	149.32 ± 27.49

Data are expressed as mean ± standard deviation. $P > .05$.

[a]Male rats had higher ALT activity than their female counterparts at $P < .0001$.

C, 10 mL/kg bwt of distilled water before weaning and free access to plain tap water after weaning ($n=20$; 9 males, 11 females); Fr, 10 mL/kg bwt of a 20% fructose solution before weaning and free access to a 20% fructose solution after weaning ($n=20$; 9 males, 11 females); ZFr, 10 mL/kg bwt of a 20% fructose solution combined with zingerone at 40 mg/kg bwt before weaning and free access to a 20% fructose solution after weaning ($n=21$; 9 males, 12 females); Z, zingerone only at 40 mg/kg bwt in 10 mL/kg bwt of distilled water before weaning and free access to plain tap water after weaning ($n=18$; 8 males, 10 females).

ALT, alanine aminotransferase; IL-6, interleukin-6; TBARS, thiobarbituric acid reactive substances; TNF-α, tumor necrosis factor alpha.

strategically targeting the neonatal period, a similar protective effect can be conferred. This beneficial effect of zingerone could have been due to its ability to enhance fatty acid oxidation via increase in hepatic PPAR activities, thereby attenuating hepatic lipogenesis.[24,25] In addition, zingerone could have prevented the fructose-induced hepatic lipid deposition (observed in this study) by attenuating hepatic *de novo* lipogenesis, which has been shown to be one of the deleterious effect of fructose on the liver.[13] Although excessive hepatic lipid content is of diagnostic importance for hepatic steatosis, the gold standard for diagnosis of NAFLD is histological assessment of hepatocytes.[32]

Histologically, NAFLD primarily begins with fatty infiltration of hepatocytes when there are >5% steatotic liver cells in the histological sections of the liver.[5] In the present study, we observed the presence of both micro- and macrovesicular steatoses in both male and female rats that received the high-fructose diet only compared with the other groups. The observed fructose-induced steatosis in both sexes was prevented by neonatal administration of zingerone in this study, meaning that zingerone had programmed protection against the hepatic steatosis secondary to high-fructose diet consumption. The findings of this study are supported by those of a previous study in older rats, which reported that zingerone administered for 8 weeks (at 100 mg/kg bwt) prevented the fructose-induced steatosis in adolescent male Wistar rats.[24] It is thus likely that zingerone protected against accumulation of lipids in the liver by downregulating hepatic lipogenic genes and receptors including ChREBP, SREBP-1, and PPARα[24] since fructose has been reported to activate hepatic expression of these genes and receptors leading to increase hepatic lipid synthesis and storage.[14] However, the current study is limited in that we did not determine how these genes were expressed. Therefore, future studies on zingerone need to explore the molecular mechanisms involved in attenuating hepatic lipid accretion.

Additionally, zingerone has been reported to attenuate fructose-induced hepatic steatosis via decrease in hepatic pathways of fatty acids uptake, synthesis, and secretion.[24] This effect of zingerone could have been responsible for the prevention of hepatic steatosis observed in this study.

Despite the presence of hepatic steatosis in the study rats, no evidence of inflammation or fibrosis was observed in both male and female rats in this study. Thus indicating that the rats in the present study did not develop advanced fatty liver disease, which is typified by steatohepatitis and parenchymal tissue damage.[24] This has contrasted the findings of Narayanan and Jesudoss[24] who observed severe steatosis with associated inflammation and fibrosis following *ad libitum* consumption of 20% fructose solution by adolescent male Wistar rats for 8 weeks. The absence of inflammation and fibrosis in the histological samples of the study rats of both sexes could be explained based on the progressive nature of NAFLD, which begins with simple steatosis before inflammation or fibrosis manifest.[14] Additionally, the age of rats at commencement of the study could also have had an impact as younger rats are less susceptible to developing fructose-induced metabolic disorders than older ones.[33] It is also noteworthy that only a small percentage (10–15%) of patients with steatosis develop inflammation and fibrosis (NASH) later in life.[3] Fatty liver could be associated with hepatocellular damage and elevated levels of the hepatic function marker, ALT.[34]

We did not observe any significant differences in the blood levels of ALT in rats in the different groups of both sexes. The lack of impact of treatments on the ALT is supported by our histological findings that did not show evidence of inflammation, which is invariably associated with elevated ALT.[12] This lack of elevation in ALT, secondary to a high-fructose diet, was also reported by a previous study after *ad libitum* consumption of 20% fructose solution by adolescent male Wistar rats for 16 weeks.[11] Studies have shown that 25% of histologically confirmed NAFLD may exist without elevation of ALT.[34] Thus, this enzyme is mainly used for the assessment of hepatic biochemical function and hepatic damage, but not to predict NALFD.[11]

Despite ALT activity not being affected by the interventions in both sexes, we observed that the activity of ALT was higher in male rats than in female rats. It is not clear whether male sex hormones are responsible for the sex difference in the ALT activity.[35] However, since ALT is also produced by muscle cells,[35] and male rats usually have higher muscle mass than females,[36] we could speculate that the higher ALT activity observed in males could be due to their greater muscle mass when compared with females. Fructose-induced fatty liver is associated with elevated blood levels of uric acid[14] due to uncontrolled phosphorylation of fructose that converts ATP to adenosine monophosphate, which is eventually broken down to uric acid.[13]

We observed that the plasma levels of uric acid were not different across the different treatments of both male and female rats in this study, despite the presence of steatosis in the hepatocytes of fructose-treated groups. In rodents, uric acid is converted to allantoin by the enzyme uricase, thereby decreasing circulating uric acid levels.[37] Humans normally do not have the enzyme uricase and are thus more prone to develop hyperuricemia than rats when exposed to triggering factors such as high-fructose diets.[37] Fructose-induced hyperuricemia is usually associated with severe forms of NAFLD than simple steatosis.[14] Additionally, Tapia *et al.*[38] demonstrated that severe metabolic derangement could be enhanced in rats when uricase is inhibited with oxonic acid. Thus, the findings of current study showing insignificant differences in uric acid concentrations could be explained by the effect of uricase in rats and the moderate NAFLD observed. Moreover, it has also been shown that fructose-induced fatty liver can develop with or without hyperuricemia.[39] Fructose-induced fatty liver can lead to oxidative stress secondary to lipid peroxidation[13] and subsequent hepatic inflammation.[40]

Although zingerone has been reported to prevent hepatic damage by inhibiting the generation of free radicals and downregulating inflammatory cytokines of hepatic inflammation,[23–25] the treatments had no effect on the levels of TBARS, IL-6, and TNF-α in male and female rats in the present study. Fakhoury-Sayegh *et al.*[11] also reported

similar findings when 20% fructose solution was consumed *ad libitum* by adolescent male Wistar rats for 16 weeks. These findings suggested that the interventions did not cause an overt proinflammatory state, a finding that can be reinforced by histological features of the rats' hepatocytes as there were no evident inflammatory foci, further confirming the presence of a nonsevere form of the disease that had not progressed to NASH.

In conclusion, neonatal administration of zingerone orally to Sprague Dawley rats prevented the long-term fructose-induced fatty liver development in both sexes. A common mantra among health care professionals is that ''prevention is better than cure.'' Targeting this critical period of developmental plasticity, with a naturally derived phytochemical may provide an alternative prophylaxis for NAFLD and hence reduce the burden of this condition on health care facilities globally. Future studies will be directed toward understanding the mechanisms of action of zingerone that resulted in these long-term protective effectives and the possible transgenerational benefits of the interventions. Additionally, the interaction of fructose with micronutrients such as copper will also be considered in future studies since fructose has been shown to alter the utilization of copper.[41]

AUTHORS' CONTRIBUTIONS

The study was designed and conducted by N.M., B.W.L., and K.H.E. N.M. produced the first draft of the article, and the remaining authors contributed in writing and revising the article. The authors have also consented to the article submission.

ACKNOWLEDGMENTS

Staff of the Central Animal Service Unit, Faculty of Health Sciences, University of the Witwatersrand are hereby acknowledged for the care of the animals. Miss Monica Gomes and Miss Hasiena Ali are also thanked for their technical assistance with the histology.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

FUNDING INFORMATION

We acknowledge the Medical Faculty Research Endowment Fund, Faculty of Health Sciences Research Committee and School of Physiology of the University of the Witwatersrand (Grant No. 001 254 8521101 5121105 000000 0000000000 5254); the National Research Foundation (NRF) Thuthuka Fund (Grant No. TTK170415227205) that provided funds for the research. Federal University Birnin Kebbi (Nigeria) and Tertiary Education Trust Fund of Nigeria are hereby acknowledged for providing funds for support of the PhD candidate.

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