THE POTENTIAL OF ZINGERONE TO PROTECT AGAINST ETHANOL-INDUCED LIVER DISEASE

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

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DECLARATION

I, **Bernice Asiedu**, student number **1889383**, declare that this thesis is my own work except otherwise acknowledged. I contributed adequately to the research findings published in the articles stated below which are within this thesis. This thesis is being submitted for the award of a Doctor of Philosophy in Physiology at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree at any other University. All the experiments were performed with approval by the Animal Ethics Screening Committee of the University of Witwatersrand.

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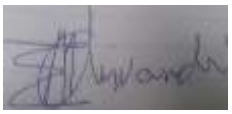
Primary supervisor: Prof Eliton Chivandi Supervisor signature:  Date: 08/05/23

DEDICATION

To my son

Bryan Jefferson Osei

(Mummy will be home soon, please hang in there)

RESEARCH OUTPUTS

Conference Presentations

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Publications

Asiedu, B., Nyakudya, T.T., Lembede, B.W., Chivandi, E. 2021. Early-life exposure to alcohol and the risk of alcohol-induced liver disease in adulthood. Birth Defects Research 113(6):451-68. doi:10.1002/bdr2.1881

Asiedu, B., Lembede, B.W., Nyakudya, T.T., Chivandi, E. 2022. Orally administered zingerone does not mitigate alcohol-induced hepatic oxidative stress in growing Sprague Dawley rat pups. Drug and Chemical toxicology. Drug and Chemical toxicology 0(0) doi: 10.1080/01480545.2022.2085740

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ABSTRACT

Alcohol can cross the placental blood-barrier and can also be secreted into breast milk. This can affect developing foetuses and/or nursing neonates negatively, thus impacting on metabolic health in early or later life. Zingerone (ZO) has anti-oxidant, anti-diabetic, anti-inflammatory, hypolipidaemic and hepato-protective properties. I hypothesised that neonatal oral administration of ZO could programme for protection against alcohol-induced metabolic derangements in suckling Sprague-Dawley (SD) rat pups mimicking human neonates that indirectly consume alcohol through their mother's breast milk.

The first experiment evaluated ZO's potential to protect suckling rat pups against alcohol-induced metabolic derangements. Seventy 10-day old SD rat pups (males = 35; females = 35) were randomly assigned to four groups and administered treatments daily from postnatal (PND) 12-21: group 1-nutritive milk (NM), group 2-1 g/kg body mass ethanol (Eth), group 3-40 mg/kg body mass ZO and group 4 - NM+Eth+ZO. Terminal body mass, blood glucose concentration, lipid profile and hepatic antioxidant status were determined. Zingerone and ethanol had no effect on pups' growth performance, blood glucose, total cholesterol, HDL- and LDL-cholesterol and hepatic thiobarbituric acid (TBARs), superoxide dismutase and catalase concentrations ($p > 0.05$). Ethanol decreased plasma triglyceride concentration in female rat pups ($p = 0.04$) but increased hepatic cytochrome P450E21 (CYP2E1) and decreased total glutathione (tGSH) concentration in male rat pups ($p < 0.05$). Zingerone increased tGSH in male rat pups ($p = 0.003$). A combination of ZO and ethanol increased ($p = 0.047$) hepatic CP2E1 concentration in male rat pups compared to control but had no effect ($p = 0.717$) on tGSH concentration. Neonatal orally administered ethanol induced hepatic oxidative stress which ZO, administered during the suckling period, failed to protect against.

In experiment II, 123 SD rat pups (males = 60; females = 63) were administered the same neonatal interventions as in experiment I but from PDN22 they were grown to adolescence

(PND45) with *ad libitum* access to normal rat chow and tap water. From PND 46-100, rats from each of the four neonatal groups were divided into two subgroups: subgroup I had tap water and subgroup II had ethanol solution as drinking fluids, for eight weeks. Body mass, feed, fluid and caloric intake were measured. Blood glucose concentration, plasma alanine transaminase and aspartate transaminase (ALT and AST) activities, adiponectin (ADP), leptin (LEP) and insulin (INS), tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and cytochrome P4502E1 (CYP2E1) concentrations were measured. HOMA-IR was computed. Visceral fat mass, hepatic fat content and histomorphometry were assessed. Hepatic TBARs and mRNA expressions of *peroxisome proliferator activator receptor-alpha* (*PPAR-α*), *sterol regulatory element binding protein 1c* (*SREBP1c*), *nuclear factor kappa beta* (*NF-Kβ*) and *TNF-α* were measured. Ethanol consumption in adulthood decreased feed and fluid intake but increased calorie intake and plasma CYP2E1 concentration ($p < 0.05$ vs control). It decreased blood glucose concentration of male rats ($p = 0.026$). A late single- and a double-alcohol hit had no effect on body and visceral fat mass of the rats ($p > 0.05$). Neonatal orally administered zingerone and ethanol and consumption of ethanol in adulthood had no effect on body mass, plasma lipid profile, adiponectin, leptin and insulin concentrations, HOMA-IR, AST and ALT activities, IL-6, TNF-α and hepatic TBARS and mRNA expression of *NF-KB* and *TNF-α* ($p > 0.05$). A late single hit with ethanol increased hepatic fat content of male rats only ($p = 0.014$). A double and or late single ethanol hit increased liver fat content in female rats ($p < 0.05$). Both a late single and double ethanol hit downregulated *PPAR-α* but upregulated *SREBP1c* expression in male and female rats ($p < 0.05$) and it caused the development of large droplet macrosteatosis. A combination of neonatal orally administered ZO and a late single ethanol hit decreased visceral fat mass of female rats ($p = 0.045$ vs control) but it did not affect the blood glucose concentration of male rats ($p > 0.05$). Neonatal orally administered ZO with either a late single- or a double-ethanol hit caused hepatic macrosteatosis, but it had no effect on mRNA

expression of *PPAR-α* of the rats ($p > 0.05$). However, neonatal orally administered ZO in combination with a late single ethanol hit did not affect *SREBP1c* expression of the rats but a combination of neonatal orally administered ZO with a double ethanol hit increased *SREBP1c* expression of female rats ($p = 0.005$).

The responses of the rats to interventions showed sexual dimorphism: ethanol consumption in adulthood decreased blood glucose concentration of male rats only and an early single ethanol hit caused microsteatosis only in female rats. Zingerone protected male rats against ethanol-induced hepatic fat accumulation. It attenuated the ethanol-induced upregulation of hepatic *SREPB1c* expression in males but not in females. Ethanol (late single and/or double hit) downregulated the hepatic *PPAR-α* expression in the rats which was mitigated by ZO. Neonatal orally administered ZO attenuated the late single- and double-hit ethanol-induced macrosteatosis in the rats. Thus, neonatal orally administered ZO can potentially be used as a prophylactic agent against ethanol-induced hepatic lipid accumulation in males and steatosis in both males and females.

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May the Almighty God continue to shine His light on our paths.

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LIST OF ABBREVIATION

AA	Acetaldehyde
Acetyl Co	Acetyl coenzyme A
ADH	Alcohol dehydrogenase
AFLD	Alcohol fatty liver disease
AH	Alcoholic hepatitis
AKT	Protein kinase β
ALD	Alcohol liver disease
ALT	Alanine aminotransferase
AMP	Adenine monophosphate
AMPK	Adenine monophosphate –activated protein kinase
ANOVA	Analysis of Variance
AREC	Animal Research Ethics Committee
AST	Aspartate aminotransferase
Bwt	body weight
ChREBP	carbohydrate-responsive element-binding protein
CYP2E1	Cytochrome P450 subfamily 2E1
DNA	Deoxyribonucleic acid
DNL	*de novo* lipogenesis
DOHaD	Developmental origins of Health and Diseases
ELISA	Enzyme-linked immunosorbent assay
Eth	Ethanol
ER	Endoplasmic reticulum
FAEE	Fatty acid ethyl esters

FASD	Foetal alcohol syndrome disorders
FBG	Fasting blood glucose
FFA	Free fatty acids
Fig	Figure
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIT	Gastrointestinal tract
H&E	Haematoxylin and Eosin
H_0	Null hypothesis
HDL-C	High density lipoprotein cholesterol
HFD	High-fat diet
HI	Alternate hypothesis
HOMA-IR	Homeostatic model of insulin resistance
IL-1β	Interleukin 1β
IL-6	Interleukin 6
iNOS	Inducible nitrogen oxide synthase
INS	Insulin
LDL-c	Low density lipoprotein cholesterol
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MDA	Malondialdehyde
MEOS	microsomal ethanol oxidising system
MetS	Metabolic syndrome
mRNA	messenger RNA
MT	Masson's Trichrome
NAD	Nicotinamide adenine dinucleotide

NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NAE	Neonatal alcohol exposure
NAFLD	Non-alcoholic fatty liver disease
NF-Kβ	Nuclear factor kappa β
PAE	Prenatal alcohol exposure
PKc	Pancreatic protein kinase
PND	Postnatal day
PPAR α	Perixosome proliferator-activated receptor α
PPAR γ	Perixosome proliferator-activated receptor γ
RNA	ribonucleic acid
ROS	Reactive oxygen species
SREBP-1c	sterol regulatory-binding protein 1c
TAG	Triacylglycerol
TC	Total cholesterol
TG	Triglycerides
TNF-α	Tumor necrosis factor α
UCP1	Uncoupling protein 1
VLDL-c	Very low density lipoprotein cholesterol
WHO	World Health Organisation
ZO	Zingerone

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CHAPTER ONE: INTRODUCTION AND JUSTIFICATION

1.0 Preview

This thesis is in six chapters. Chapter one introduces the reader to the challenges wrought in suckling neonates exposed to alcohol through milk from breastfeeding mothers and proposes and justifies an intervention that can be used to prevent the challenge. The chapter clearly states the study aim, objectives and hypothesis. Chapter two situates the study within bodies of existing literature, discussing themes relevant to understanding ethanol's metabolic programming potential and the use of zingerone to potentially prevent its (ethanol) adverse effect. The chapter explores ethanol's peri-and preconception, *in-utero* and postnatal potential to programme adverse metabolic health outcomes. The mechanisms of alcohol-induced metabolic dysfunction and liver disease is explored, and the chapter concludes by describing the potential of using zingerone as a prophylactic agent to mitigate alcohol-induced metabolic derangements and liver dysfunction. Chapter three gives a narrative (mini-introduction, methods, data analyses, results and discussion) of the first experiment that investigated the short-term potential protective effect of zingerone against alcohol-induced oxidative stress and its effects on oxidative injury and associated metabolic derangements in suckling rat pups mimicking human neonates indirectly consuming alcohol from the mother’s breast milk. Chapter four gives a narrative on the potential of neonatal orally administered zingerone to programme for protection against the development of alcohol-induced metabolic dysfunctions in adult Sprague-Dawley rats. The chapter’s introduction brings to fore the 'single-hit' and 'double-hit' programming of alcohol and its association with metabolic dysfunction and then gives a description of the methodology, narrates and discusses the study findings and draws appropriate conclusions regarding the potential of neonatal oral administration of zingerone to programme for protection in adulthood. The fifth chapter gives a narrative on the evaluation of the potential of neonatal orally administered zingerone to programme for protection against

alcohol-induced fatty liver disease (AFLD) in adulthood. The study investigated the potential long-term beneficial effects of the zingerone against the development of AFLD in adult rats subjected to a single late and double ethanol hit. Different assays that have a bearing on hepatic health were done in a bid to evaluate the efficacy of zingerone as a prophylactic agent against alcohol-induced AFLD. A detailed discussion and conclusion are presented as well as the potential practical applications of the study. Chapter six gives a summary of the major findings and conclusions drawn from the study. It also highlights limitations of the study and proposes the direction and recommendations future studies could explore. Chapter seven is the list of references, appendices including ethics certificate and supplementary data.

1.1 Introduction

Alcohol use during pregnancy and lactation can have detrimental effects on the child (Caputo et al., 2016; Gibson & Porter, 2018). This has led to strict recommendations regarding alcohol consumption, particularly during pregnancy (WHO, 2014). Despite these recommendations, several studies report a reduced maternal alcohol intake during pregnancy and a return to pre-pregnancy levels or at least higher consumption levels than during pregnancy shortly following birth (Tung et al., 2020). Alcohol quickly passes into breastmilk via passive transport and can be transferred to the breastfeeding infant (D'Apolito, 2013). The prevalence of alcohol consumption among nursing women is reported to range from 36%-83% (Haastrup et al., 2014). The most commonly reported reasons are societal pressure and the belief that only 'strong' and large doses of alcohol can negatively affect the child (Popova et al., 2022). Meanwhile, studies report that excessive maternal alcohol consumption contributes to reduced abstract reasoning and motor development (Little et al., 2002; Gibson & Porter, 2018) and increases the risk of foetal alcohol syndrome in offspring (May et al., 2016). Additionally, the indirect consumption of alcohol by suckling neonates through maternal breast milk has been shown to mediate increased methylation of the dopamine receptor that could cause adverse health outcomes (Fransquet et al., 2017).

Neonatal programming is a phenomenon whereby the interaction between inherited (endogenous) and environmental (exogenous) factors results in epigenetic changes that can cause temporary or permanent alterations in physiological development and function (Agosti et al., 2017). Subsequently, these alterations can lead to either improved survival or ill-health during the immediate neonatal developmental phase and or later in adult life (Agosti et al., 2017). *In vivo* studies show that neonatal alcohol exposure causes oxidative hepatic injury due to the direct metabolism of alcohol by the liver (Tavares do Carmo et al., 1996; Ojeda et al., 2009; de Freitas et al., 2014). Prenatal studies also show foetal exposure to alcohol via substrate

exchange at the placenta causes intrauterine growth restriction (IUGR) associated with activation of the lipid transcript genes sterol-regulating element-binding protein (*SREBP1-c*) and fatty acid synthase (Shen et al., 2014). These lipid regulating genes may remain activated into adulthood leading to the development of dyslipidaemia and obesity (Magee et al., 2008). Furthermore, early-life alcohol exposure through maternal and paternal alcohol consumption can induce alcohol use disorder in offspring later in life (Chang et al., 2019; Gaztañaga et al., 2020). Thus early-life alcohol exposure may be a significant independent risk factor for metabolic dysfunction, poor liver health and alcohol use disorders in childhood and adulthood.

Long-term excess alcohol consumption causes alcohol-induced liver disease (ALD) and metabolic disorders, including insulin resistance, deranged glycaemic control and hypertriglyceridaemia (Hosseini et al., 2019; You & Arteel, 2019). The combination of insulin resistance and any two or more metabolic disorders or abnormalities such as hypertriglyceridemia, hypertension lead to the development of metabolic syndrome (Nilsson et al., 2019). Ji (2008) reports that ethanol induces endoplasmic stress and cyclic AMP-responsive element-binding protein H (CREBH). The induction of CREBH up-regulates sterol regulatory element binding transcription factor 1 c (*SREBP-1c*) (Nakagawa & Shimano, 2018) which then interacts with lipid droplets (LD) membrane proteins promoting lipid droplet formation and steatosis (Gao et al., 2017). Steatosis is also associated with an increased ratio of reduced nicotinamide adenine dinucleotide to oxidised nicotinamide adenine dinucleotide resulting in the inhibition of β-oxidation of fatty acids, which enhances triglyceride and very-low-density lipoprotein synthesis (Liu, 2014). Excessive alcohol consumption therefore contributes to ALD and metabolic dysfunction. Based on my thorough literature search, most studies that investigated the impact of exposure to alcohol in early-life in adult rats used a high-fat diet as the second insult (Akison et al., 2019). To my knowledge no data exist on the double hit insult of alcohol resulting from early-life exposure and alcohol consumption in adulthood.

Alcohol-induced liver disease and metabolic syndrome (MetS) cause significant morbidity and mortality and place a heavy burden on the healthcare system (Rehm et al., 2013; Guembe et al., 2020) due to the lack of ideal therapeutic treatment and management strategies in addition to the requirement for multiple targets for preventive interventions (Buzzetti et al., 2017; Nilsson et al., 2019). The theory of the developmental origins of health and disease (DOHaD) opens an opportunity to intervene, during the neonatal developmental phase, in the prevention of the development of adverse health conditions by administering beneficial pharmacological agents that prevent the impact of early, late or double insults on offspring (Hsu et al., 2021). Rats are altricial species that provide a critical window of opportunity, during lactation, for positive neonatal programming (Rabadán-Diehl & Nathanielsz, 2013). Recent research has demonstrated that consuming phytochemicals during the neonatal period can prevent the development of adverse health conditions induced by neonatal or adult programming effects (Lembede et al., 2018; Nyakudya et al., 2018; Mukonowenzou et al., 2021; Muhammad et al., 2021a).

Zingerone, a vanillyl acetone, is obtained by drying ginger or by thermal degradation of gingerols or shogaols via the retroaldo reaction (Zhang et al., 2012). It possesses anti-obesogenic, anti-diabetic, anti-oxidant, anti-inflammatory, hepato-protective and anti-microbial properties to name but a few (Ahmad et al., 2015; Mani et al., 2017). Neonatal orally administered zingerone at 40 mg/kg body weight attenuated and reversed fructose-induced hepatoxicity and metabolic derangements in adult rats (Muhammad et al., 2021a, Muhammad et al., 2021b). Zingerone's cardiometabolic and hepatoprotective properties might be due to its ability to inhibit xanthine oxidase, stimulate lipolytic pathways and downregulate apoptotic proteins involved in the pathophysiology of metabolic dysfunction and ALD (Dunn and Shah, 2016). All these properties make the neonatal oral administration of zingerone a potentially

feasible strategic intervention that can prevent the development of alcohol-induced liver disease and associated metabolic disorders in early and adult life.

1.2 Rationale

Excessive alcohol consumption is a public health concern as it contributes to over 200 diseases (Leslie et al., 2015) and annually it accounts for 3 million deaths globally (Leslie et al., 2015). Alcohol consumption accounts for 20% to 50% of the prevalence of liver cirrhosis (WHO, 2018). In their review, Rehm et al. (2013) reported that alcohol-attributable liver cirrhosis was the cause of 0.9% (0.7% for women and 1.2% for men) of all global deaths and 0.6% (0.4% for women and 0.8% for men) of all global disability-adjusted life years; 47.9% of all liver cirrhosis deaths (46.5% for women and 48.5% for men) and 46.9% of all liver cirrhosis disability-adjusted life years (44.5% for women and 47.9% for men). Alcohol-attributable liver cancer accounted for 80 600 deaths globally (Rehm et al., 2013). According to WHO report, ALD is the commonest cause of cirrhosis in the western world. It is currently one of the ten most common causes of death despite the burden of alcohol-related liver disease being entirely preventable (WHO, 2018). Most studies on alcoholic liver disease are conducted in adult humans and rats because ALD and MetS usually occur in adulthood. However, emerging evidence reveals that exposure to alcohol during the period of increased developmental plasticity can also lead to deleterious health outcomes including ALD and MetS (Agosti et al., 2017). The protection against the development of adverse alcohol-induced health outcomes and the conferment of positive health outcomes later in life can also be programmed by orally administering substances with health beneficial properties during the critical periods of developmental plasticity (Ajah et al., 2022; Mohammed, 2022). The antioxidant, anti-inflammatory, anti-apoptotic, hepato-protective and anti-hyperlipidaemic properties of zingerone makes it a feasible intervention that can be administered during suckling to

potentially programme for protection against early or late ‘single-hit’ or ‘double-hit’ alcohol-induced fatty-liver disease. Currently, there is no literature pointing to the potential of neonatal orally administered zingerone to programme for protection against early or late single-hit or double-hit ethanol-induced liver disease.

1.3 Aim

The study sought to determine the potential protective effect of orally administered zingerone during the suckling period to rats against developing early or late 'single-hit' or 'double-hit' ethanol-induced fatty liver disease in adulthood.

1.3.1 Specific objectives

Specifically, the study determined the effects of neonatal orally administered zingerone on:

i) the growth performance, organ morphometry, hepatic oxidative stress and hepatic and plasma lipids induced by ethanol in suckling rats.
ii) the potential protective effects of zingerone against alcohol-induced adverse effects by evaluating outcomes on glucose and lipid homeostasis.
iii) the potential of protective effects of zingerone against alcohol-induced inflammation, oxidative status and alcohol-induced liver disease in adult rats.

1.4 Hypotheses

Two main experiments were carried out and each had specific hypotheses statements.

1.4.1 Experiment 1 hypothesis:

H_0: Neonatal orally administered zingerone does not protect suckling rat pups against ethanol-induced changes in growth performance, organ morphometry, hepatic and plasma lipids and hepatic oxidative stress.

H_1: Neonatal orally administered zingerone protects suckling rat pups against ethanol-induced changes in growth performance, organ morphometry, hepatic and plasma lipids metabolic and hepatic oxidative stress.

1.4.2 Experiment 2 hypothesis

H_0: Neonatal orally administered zingerone does not protect adult rats against the development of ethanol-induced glucose and lipid dyregulation, inflammation, oxidative derangements and alcohol-induced fatty liver disease by either neonatal or adult "early and late single hit" and/ or a "double hit" alcohol insult.

H_1: Neonatal orally administered zingerone protects adult rats against the development of ethanol-induced glucose and lipid dysregulation, inflammation, oxidative derangements and alcohol-induced fatty liver disease by either neonatal or adult "early and late single hit" and/or "double hit" alcohol insult.

The next chapter, chapter 2, presents pertinent literature regarding the prevalence of pregnant and nursing mothers that indulge in alcohol consumption and alcohol's programming effect in both early and adult life. The chapter elucidates current knowledge on alcohol-induced liver disease and its mechanistic pathways. It concludes with a review of the pharmacological properties of zingerone that make it a potential feasible intervention against alcohol-induced liver disease and associated metabolic derangements.

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CHAPTER TWO-LITERATURE REVIEW

2.0 Alcohol consumption among pregnant and lactating women

Alcohol consumption in pregnant women is a significant public health concern due to the negative impact on the developing foetus and the mother (Lamy et al., 2019; Seymour et al., 2019). It can cause miscarriage, premature and stillbirths, IUGR, low birth weight and congenital abnormalities (Hoyme et al., 2016; Myers et al., 2018; Sundermann et al., 2019; Mitchell et al., 2020). Additionally, it causes foetal alcohol syndrome disorders (FASD) and other neurodevelopmental deficits (Hoyme et al., 2016). Due to the adverse effects of alcohol, heath guidelines advocate for abstinence from its consumption by reproductive women before or during pregnancy (Chola et al., 2017; CDC, 2020). Clinical studies have and continue to show that alcohol drinking in pregnancy is only harmful when consumption is heavy (Robinson er al., 2010; Callinan & Room, 2012). On the other hand, meta-analyses of the sparsely available studies on the effect of low-to-moderate alcohol consumption in pregnancy are inconclusive (Mamluk et al., 2017; Wilson et al., 2017).

The exact prevalence and amount of alcohol consumed among pregnant and lactating women is not precise because it is often under-reported due to social desirability bias, religious beliefs, seasonal and geographical variations, among other reasons (Addila et al., 2020). Globally, about 10% of pregnancies are exposed to alcohol (Popova et al., 2017). Countrywide data of pregnant women collected from the United States of America between 2015 and 2017 showed a prevalence of 11.3% (Denny et al., 2020). An estimated 20.83% of pregnant women consume alcohol in sub-Saharan Africa (Addila et al., 2020). Popova et al. (2016), in the WHO Africa Report, contend that a little over 18.25% of pregnant women consume alcohol. In South Africa, the national household data suggested that among women who drank alcohol, 15.6% are heavy drinkers and 30.5% are binge drinkers (Martinez et al., 2011). In the Western Cape Province

of South Africa, where FASD rate is among the highest globally (May et al., 2005); 24% of the controls (women without FASD children) admitted to drinking at least six drinks per week during pregnancy (May et al., 2005).

Data on the prevalence of alcohol consumption in lactating mothers is limited. Although most heavy alcohol-drinking women abstain or reduce alcohol consumption when pregnant, the majority (80%) of them return to sober alcohol drinking habits during breastfeeding (Tran et al., 2015). According to Rebhan et al. (2009), women are more careful with their alcohol intake after birth, but alcohol intake increases as the child grows older. In the study by Rebhan et al. (2009), only 5% (n = 58) drank 1-3 glasses of alcoholic beverages in the first three months postpartum but increased to about 10% by the sixth month and 15% by the seventh month. A meta-analysis of 8 studies estimated that the prevalence of alcohol use among lactating women was 36-83% (Haastrup et al., 2014). In South Africa, a FASD study revealed that 70.1% of the women sampled (1047) consumed alcohol during breastfeeding (May et al., 2016). Despite the aforementioned exposure to alcohol during the neonatal growth phase, exposure to alcohol through breastmilk is a matter of contention hence in the next section below is a description of how alcohol enters breastmilk and finds its way to the breastfeeding infant.

2.1 Transfer of alcohol into the breastmilk

It is pertinent to unravel how breastmilk forms in order to create a platform necessary to understand the mechanism of the transfer of alcohol into breastmilk and to the breastfeeding infant. The mammary gland (breast), the organ that produces breastmilk, contains 15-20 lobes that diverge from the nipple (D'Apolito, 2013). A lobe comprises 20 to 40 lobules and each small milk duct contains 10-100 alveoli (D'Apolito, 2013). It is the mammary gland aveoli that synthesise milk (D'Apolito, 2013). Extending from the alveoli are small ducts that empty into

a sizeable lactiferous duct. Each lactiferous duct has a lactiferous sinus that narrows into the nipple for excretion of the breastmilk (D'Apolito, 2013). During pregnancy, estrogen mediates massive proliferation and expansion of the breast's alveoli units and progesterone causes an increase in the size and number of the lobes, lobules and alveoli (Hale, 2018). During pregnancy, increased estrogens and progesterone concentration inhibit the secretion of prolactin, a hormone that induces lactation (Alex et al., 2020). At parturition, estrogen and progesterone concentrations drop drastically allowing signals to be transmitted to the anterior pituitary for prolactin release (Alex et al., 2020). Additionally, the elimination of the placental lactogen helps to promote the prolactin-mediated production of breastmilk (Alex et al., 2020). The stimulation of nipples by the suckling infant facilitates the maintenance of postpartum prolactin concentrations (Hale, 2018).

Alcohol passes into breastmilk through two transport mechanisms: passive or facilitated diffusion (D'Apolito, 2013). Non-bound, low molecular weight substances pass from a higher concentration to a lower concentration. In the case of alcohol, the high alcohol concentration levels in the maternal plasma create a concentration gradient that allows it to pass into the breastmilk. Lactocytes line the breast alveoli, and at the basal membrane are capillaries that transport nutrients, fats, proteins and other components from the maternal plasma into the breastmilk (D'Apolito, 2013). The lymphocytes also transport immunoglobulins into the breast (Palmeira & Carneiro-Sampaio, 2016). During the early hours of lactation, the lactocytes are small and have significant gaps that allow the passage of these substances into breastmilk. Immediately following the synthesis and secretion of colostrum, lactocytes become bigger, and the gaps become smaller, substantially reducing a significant amount of substances from passing into the breastmilk (D'Apolito, 2013). Despite these structural changes, alcohol can pass through the gaps because of its small molecular weight (46 Da), higher acid-base dissociation increased mythylation of the dopamine receptor 4 and reduced acetylation of

TRB3 (tribbles 3) and PTEN (phosphatase and tensin homolog deleted on chromosome ten) (15.9) and its non-protein bound nature (D'Apolito, 2013). Exposure to alcohol during the early postnatal growth phase can result in that can affect physiological and metabolic function as it is a period of developmental plasticity (Fransquet et al., 2017; Ellsworth et al., 2018).

2.2 Neonatal programming

Neonatal programming is produced by deviations from normal development as a result of "insult" during early postnatal life (Agosti et al., 2017). Experiences of life events early in life can have significant long-term effects on physiological development (McEwen, 2003). The effect of neonatal programming can be immediate or observed later on in adulthood. In conditions where the impact of a single stimulus early in life or in adulthood is evident is referred to as the "single hit" theory (Tamashiro & Moran, 2010). This single hit may be the first hit for a double/multiple hit effect. In the "multiple and/or double-hit theory", the effect of an insult is seen upon multiple hits or a second hit, respectively (Stewart et al., 2013). In the case of a "double and/or multiple-hit theory" the first hit usually remains latent until another stimulus is applied. The neonatal period is a "critical developmental window" in which organisms are susceptible to the environmental chemicals that may affect the organismal phenotypic expression (Agosti et al., 2017). Such windows represent a form of developmental phenotypic plasticity and result from the interaction between genotype and environment. The period of developmental plasticity spans from perinatal to early postnatal life (Chen & Nyomba, 2004; Ellsworth et al., 2018; Gårdebjer et al., 2018). When taken orally, many substrates, including alcohol, can elicit neonatal programming (Nyakudya et al., 2018; Conner et al., 2020; Muhammad et al., 2021a).

2.3 Ethanol and neonatal programming

Ethanol is a well-known teratogen (Hoyme et al., 2016) whose effect on the foetus has received much attention. Foetal alcohol syndrome disorders which may be observed in the offspring of women who consumed alcohol during pregnancy (Conner et al., 2020), are characterised by abnormalities in foetuses and the early neonatal growth phase. The abnormalities range from " prenatal and postanatl growth retardation, brain abnormalities (microcephaly), cognitive deficits and behavioral anomalies such as attention-deficit/hyperactivity disorder minor facial features including short palpebral fissures, a thin upper lip, and a long, smooth philtrum. The spectrum spans from foetal alcohol syndrome, partial foetal alcohol syndrome, alcohol-related neurodevelopmental disorder and alcohol-related congenital disabilities (Hoyme et al., 2016). The average ethanol dose in a pregnant woman associated with FASD is 2.2 g/kg/d (Willhite et al., 1988). While FASD is associated with maternal alcohol consumption, heavy paternal alcohol consumption also elicits adverse developmental outcomes in offspring (Hoyme et al., 2016; Chang et al., 2019; Conner et al., 2020). Paternal alcohol consumption before conception can induce dramatic neocortical gene expression and subtle changes in neocortical connectivity that extend to second and third filial generation (Zuccolo et al., 2016 ; Conner et al., 2020).

Alcohol consumption during the perinatal period can programme for the development of adverse outcomes in the heart of children born to alcoholic mothers (Caputo et al., 2016). Studies found a 1.6-fold likelihood of having a child born with d-transposition of the great arteries, a condition that causes the aorta and the pulmonary arteries to be reversed in offspring of mothers who drank alcohol while pregnant (Henderson et al., 2007; Caputo et al., 2016). Furthermore, *in utero* alcohol-induced neurotoxicity has been shown to cause hypoplasia, agenesis, kidney hydronephrosis, glomerulosclerosis, hyperbilirubinemia in the liver and

enteric neuropathy (Vasiliauskas et al., 1997; Hofer & Burd, 2009; Caputo et al., 2016). Early life exposure (*in utero* or early postnatal period) to alcohol affects the potency and differentiation of mesenchymal stem cells (Nakhoul et al., 2017) that are responsible for tissue repair resulting in increased susceptibility to disease later in life (Nakhoul et al., 2017). *In vivo* studies have shown that exposure to alcohol *in utero* and or in early postnatal life can programme offspring for non-alcoholic fatty liver disease, dyslipidaemia and insulin resistance (Chen & Nyomba, 2004; Shen et al., 2014; Nguyen et al., 2019).

2.4 Ethanol and metabolic programming

Evidence shows that exposure to alcohol in early-life during the critical period of developmental plasticity affects body composition, glucose and lipid metabolism as well insulin and lipid signaling (Akison et al., 2019). Human and preclinical studies have shown that ethanol exposure at peri-conception, prenatal and early postnatal life programmes metabolic dysregulation in offspring (Castells et al., 1981; Chen & Nyomba, 2003a, 2003b; Yao & Nyomba, 2008; Carter et al., 2012; Yao et al., 2013; Amos-Kroohs et al., 2016). While many studies focused on metabolic programming at the foetal stage and in addition to the nursing phase, only two studies (Do Carmo et al., 1999; Chen & Nyomba, 2004) explored the lactation period exclusively.

Developmental origins of health and adult disease (DOHaD) are primarily associated with *in utero* environmental factors (Ellsworth et al., 2018). However, emerging evidence suggests that the period before conception as well as early embryogenesis is crucial in DOHaD (Velazquez et al., 2019). Peri-conception and pre-conception are periods during which organisms are vulnerable to environmental factors such that changes via disruptive or adaptive compensatory responses may persist throughout an organism's lifespan (Velazquez et al.,

2019). At peri-conception, parental genomes supersede the embryonic genome, and the establishment and differentiation of a new cell require significant epigenetic, cellular and metabolic activity that occurs in the maternal oviduct and uterine lumens (Gardner & Harvey, 2015; Lim et al., 2016; White et al., 2016). Maternal lifestyle factors such as alcohol consumption can modify the oviduct and uterine transport system, affecting the luminal compartment's nutrient composition and the natural environment experienced by the early embryo (Eckert et al., 2012; Jordaens et al., 2017). Alcohol can diffuse into watery compartments, including the uterus and oviduct and cause damage to the developing embryo (Gardebjer, 2015). According to Mitchell (1994) ethanol advances the implantation of blastocytes which can lead to post-implantation loss without affecting the rat. Furthermore, ethanol can increase blood flow to the blastocyte implantation site (Mitchell, 1994). However, another research team found ethanol-exposed rat dams to have fewer embryos on embryonic day 12, reduced implantation site numbers, increased resorption, delayed embryonic development and abnormal neural tube closure (Coll et al., 2011). Clearly, the pre-implanted embryo is sensitive to ethanol and can become susceptible to epigenetic changes; mechanisms known to play a role in disease programming (Ikeda et al., 2012).

Alcohol passes directly into the placenta (Zhang et al., 2005) and can affect the developing cells and tissues of the foetus. In the developing foetus alcohol elimination rate is 3% to 4% slower than in an adult (mother) resulting in its accumulation in the amniotic fluid (Heller & Burd, 2014). Consequently, the reduced alcohol elimination rate coupled with relatively lower activity of the foetal enzyme CYP2E1, the enzyme that detoxifies alcohol in the foetus cultivates a local microenvironment that mediates an increase in oxidative stress resulting in foetal organ damage (Joya et al., 2015). During the prenatal growth phase, exposure to alcohol causes epigenetic changes, which can induce adverse metabolic outcomes (Fransquet et al.,

2017; Akison et al., 2019). For example, Yao et al. (2013) demonstrated that prenatal exposure to 4 g/kg ethanol caused alterations in histone acetylation that resulted in glucose intolerance in adulthood in SD rats.

Prenatal developmental programming events have received extensive attention (Probyn et al., 2013; Yao et al., 2013; Shen et al., 2014). The early postnatal critical window is less studied, although the lactation period is a crucial time of development, differentiation and reorganisation of metabolic tissues (Feng et al., 2013; Scaglia et al., 1997). This period can have an independent yet equally significant impact on metabolic programming (Ellsworth et al., 2018; Hafner et al., 2019). Development of the respiratory, cardiovascular and endocrine systems occur in the early neonatal phase while the development and maturation of the hepatic, haematological and renal systems occur in the late neonatal stage (Sgarbieri et al., 2017; Sharma et al., 2011). In rats, differentiation and maturation of liver cells occur between postnatal days 9 to 28, equivalent to 0-5 years in humans (Alexander et al., 1997). Hence a dietary insult such as indirect alcohol consumption during the postnatal growth phase can affect the development and function of the liver leading to an array of metabolic dysfunctions.

2.5 Early-life exposure to alcohol, alcohol-induced metabolic and liver disease in adulthood

Preconception and prenatal alcohol consumption by mothers cause marked neuronal and behavioural effects in offspring typified by characteristics of alcohol use disorder (Chotro et al., 2007; Chang et al., 2012; Nizhnikov et al., 2016). Importantly, studies show that exposure to alcohol during the prenatal development phase increases ethanol consumption and preference in adult offspring (Gaztañaga et al., 2020). Furthermore, it promotes behavioural changes, for example, hyperactivity, anxiety, impulsivity and cognitive dysfunction (Carneiro

et al., 2005; Cullen et al., 2013; Hoyme et al., 2016). These behavioural changes impact judgment and influence alcohol consumption. Therefore, exploring whether exposure to alcohol during the lactation period programmes offspring for alcohol-induced metabolic dysfunction and liver disease in adulthood is pertinent.

Tsukamoto's review of substances used as 'second hit' of ALD includes nutritional, genetic, hepatitis C virus, hemodynamic and pharmacological insults (Tsukamoto et al., 2009). Most rodent studies that modelled the programming effect of perinatal alcohol exposure used ethanol as the first hit, but the second hit was always a different stimulus, usually a high-fat diet (Tsukamoto et al., 2009; Shen et al., 2014; Nguyen et al., 2019; Chen et al., 2020). To my knowledge, no study has used alcohol alone as the "second hit" for ALD. However, the evidence provides that alcohol consumption depending on the dose and duration can produce a similar effect as a high-fat diet (Tsukamoto et al., 2009). Acetaldehyde and malondialdehyde, by-products of oxidative stress resulting from chronic alcohol consumption, efficiently serve as a "second hit" in ALD and metabolic dysfunction (Tuma, 2002). Early-life exposure to alcohol can stimulate an early involuntary onset of alcohol use. Given that early alcohol intake increases the propensity to a continued history of alcohol consumption; this may lead to alcohol use disorder and eventually alcohol-induced metabolic and liver disease in adulthood (Hagström et al., 2018).

2.6 The metabolism of alcohol

The liver eliminates consumed alcohol from the body through the oxidation of alcohol into acetaldehyde and acetate. Dunn and Shah (2016) indicated that the metabolism of ethanol occurs in the primary liver parenchymal (hepatocytes) cells. These cells express the highest activities of alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1); enzymes

that break down ethanol to acetaldehyde (Singal et al., 2018). Alcohol dehydrogenase, located in the cytosol and CYP2E1 found in the smooth endoplasmic reticulum of hepatocytes, oxidise alcohol to acetaldehyde (Singal et al., 2018). In addition to ADH and CYP2E1, the major enzymes that catalyse alcohol metabolism, catalase plays an accessory role in the detoxification of ethanol by using H_2O_2 to oxidise ethanol to acetaldehyde (Aragon et al., 1992).

Alcohol metabolism and elimination from the body occur by two different pathways: oxidative and non-oxidative pathways. As shown in **Figure 2.1** oxidative alcohol metabolism occurs when ADH metabolises alcohol into acetaldehyde, a highly toxic and reactive molecule (Stickel et al., 2017). This ADH-catalysed process accompanies the reduction of nicotinamide adenine dinucleotide (NAD) into NAD hydrogen (NADH). The microsomal ethanol oxidising system, specifically CYP2E1, is induced under elevated or chronic consumption resulting in the oxidation of the excessive amount of alcohol to acetaldehyde (Ji, 2008a; Leung & Nieto, 2013). Catalase, located in the peroxisome, also plays an accessory role in metabolising ethanol to acetaldehyde (Aragon et al., 1992). The acetaldehyde produced is further oxidised in the mitochondria by the enzyme aldehyde dehydrogenase 2 ($ALDH_2$) to produce acetate and a second NADH molecule (Lemasters et al., 2012; Zakhari, 2013). The acetate is released into the bloodstream and metabolised by peripheral tissues to generate energy with carbon dioxide and water as by-products.

The non-oxidative pathway of ethanol metabolism is the esterification of ethanol and fatty acids and/or fatty acyl-CoA into fatty acid ethyl esters [(FAEE), Waszkiewicz et al., 2012]. This pathway oxidises about 1% of ethanol by FAEE synthase enzymes found in the liver, pancreas, heart, and brain cytosol and microsomes (Osna et al., 2017; Waszkiewicz et al., 2012). The metabolites from alcohol metabolism are potential inducers of epigenetic changes via various mechanisms, such as elevated NADH/NAD ratios and ROS levels (Mandal et al., 2017). These metabolites modify DNA and histone by causing hypomethylation (Mandal et al., 2017).

Additionally, the critical lipid transcription factors, *peroxisome proliferator-activated receptor* (*PPAR-α*) and *sterol regulatory element-binding protein 1c* (*SREBP1c*) are also affected by the epigenetic changes (You & Arteel, 2019). Alcohol up-regulates *SREBP1c* (Liangpunsakul, 2015) and down-regulates *PPAR-α* (Nan et al., 2013).

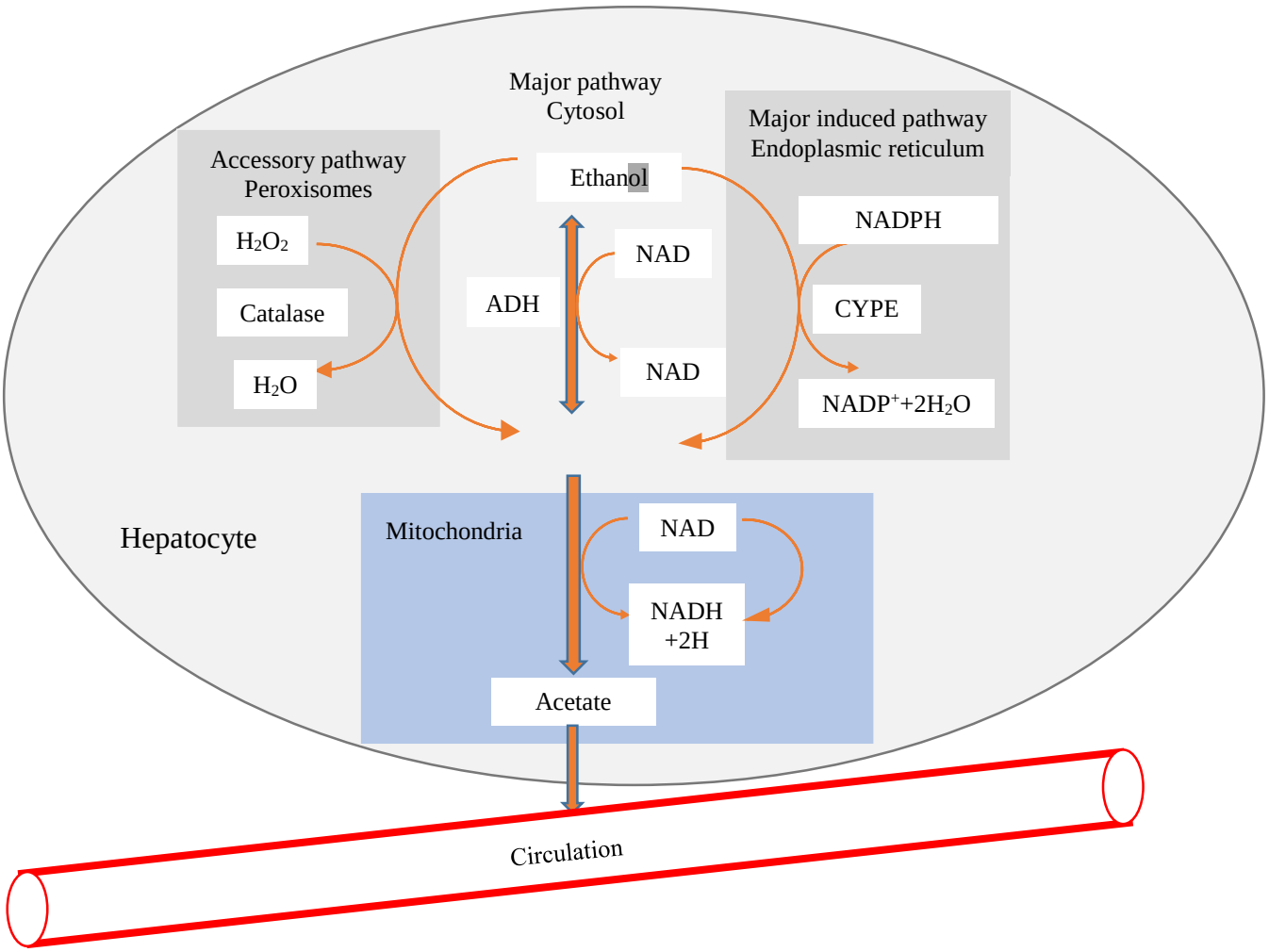

Figure 2.1: **Major and minor ethanol-oxidising pathways in the liver**

Source: Osna et al., 2017

The physiological and biochemical effects of alcohol largely depend on the interplay between its absorption, distribution and elimination (You & Arteel, 2019). Usually, following oral

ingestion, alcohol absorption is primarily via passive diffusion in the small intestine (Person, 1991). A multiplicity of factors among the nature and concentration of the alcoholic beverage, the rate of ingestion, the fed state of the person, the nature and composition of food, the rate of gastric emptying, in addition to genetic and environmental factors influence the rate of alcohol absorption (Ramchandani et al., 2001) and its impact.

2.6.1 Effect of ethanol on glucose metabolism

The effect of ethanol on carbohydrate metabolism is dependent on the eating state of the individual. Ethanol causes severe hypoglycaemia primarily by reducing hepatic glucose production through inhibition of gluconeogenesis in individuals whose glycogen stores have been depleted by fasting (Jang and Koh, 2012). However, in individuals who are fed, the effect remains debatable (Ji, 2008). The debate revolves around three points of arguments: that ethanol consumption is associated with diminished, improved, or unchanged glucose tolerance depending on the pattern and quantity of alcohol consumed (Ji, 2008a). Among human subjects, ethanol abuse has been associated with overt diabetes mellitus which resolved with abstinence from alcohol (Hodge et al., 1993; Ji, 2008). Shelmet et al. (1988) in their study on acute effects of ethanol on the metabolism of an intravenous glucose load reported that ethanol decreased total body fat and protein oxidation by 79% and 39%, respectively and almost completely abolished the rise in carbohydrate oxidation seen in healthy young males. Furthermore, ethanol reduced the basal rate of glucose appearance and its disappearance by 30% and 38%, respectively (Shelmet et al., 1988). It potentiated glucose-stimulated insulin release by 54%, and had no effect on glucose tolerance (Shelmet et al., 1988). In hyperinsulinaemic-euglycaemic clamp studies, ethanol caused a 36% decrease in glucose disposal (Shelmet et al., 1988). Thus, Shelmet et al (1988) concluded that ethanol was a preferred fuel preventing fat, and to a lesser degree carbohydrate from being used by the body.

Nguyen et al. (2012) reported that ethanol reduced insulin secretion by interfering with muscarinic signalling and pancreatic protein kinase (PKc) activation and that it also reduced insulin content and caused endoplasmic (ER) reticulum stress. Additionally, chronic alcohol consumption has been associated with increased adiposity and insulin resistance; risk factor for type 2 diabetes mellitus (Wan et al., 2005; Zhao et al., 2009). Zhao et al. (2009) reported that alcohol drinking is a conditional aetiological factor for diabetes mellitus and excessive ethanol intake is negatively associated with both insulin sensitivity and β-cell mass.

2.6.2 Alcohol and the liver-adipose axis

Like humans, adipose tissue is a multi-depot organ displaying metabolic heterogeneity and intrinsically different in functionality (Chusyd et al., 2016). There are 2 main types of adipose tissue, white adipose tissue (WAT) and the brown adipose tissue (BAT) (Chusyd et al., 2016). The BAT, commonly found in neonates, is optimized for generating heat by metabolizing fatty acids and glucose during adaptive or non-shivering thermogenesis (Scheja & Heeren, 2019). Brown adipocytes contain uncoupling protein 1 (UCP1), which mediate the proton transfer into the mitochondrion matrix to uncouple fatty acid oxidation in the mitochondrion, thereby mediating heat instead of ATP generation (Cannon & Nedergaard, 2004). The BAT is activated by cold exposure, exercise and calorie restriction (Scheja & Heeren, 2019). Ethanol also disrupt BAT lipid metabolism by increasing the expression of *UCP1* or by inducing hypothermia (Huttunen et al., 1998; Yoshimoto et al., 2004; Blaner et al., 2017). Consequently, BAT activation counteract body weight gain, obesity and metabolic disease through energy expenditure (Marlatt & Ravussin, 2017).

The WAT has three main functions: energy storage, triglyceride hydrolysis for energy support under negative energy balance and adipokine secretion (Kema et al., 2015). Adiponectin,

leptin, resistin, TNF-α, IL-6 and monocyte chemoattractant protein-1, adipokines derived from WAT, are known biomarkers of inflammation (Liang & Ye, 2019). Chronic alcohol consumption increases the density of macrophages in adipose tissue (Souza-Smith et al., 2016) and the expression of inflammatory cytokines and chemokines (Meroni et al., 2018). This creates an inflammatory milieu as occurs in high-fat diet-induced obese rodents (Souza-Smith et al., 2016). However, the difference is that with alcohol-induced inflammation, there is no increase in adipocyte size as seen in metflammation characteristics of obesity (Gregor & Hotamisligil, 2011). While hepatocytes remain the primary site of ethanol metabolism, adipocytes in adipose tissue also express ALD and CYP2E1 (Sebastian et al., 2011). The activation of CYP2E1 in response to heavy/chronic alcohol consumption is associated with the induction of oxidative stress in adipose tissue, leading to impaired adipose tissue function and secretion of adiponectin and ethanol-induced adipocyte apoptosis (Souza-Smith et al., 2016). Studies show that with heavy chronic alcohol consumption, adiponectin levels decrease (Chen et al., 2007; Yu et al., 2010). Contrary, alcohol consumption may increase circulating ADP in plasma, but its signalling may be impaired (Xu et al., 2011). At the early stages of ALD, both circulating and local production of TNF- α increase; however, increased TNF- α suppresses adiponectin production (Lin et al., 1998). Therefore, the ratio of adiponectin/ TNF- α has been suggested as a helpful marker in the management of ALD.

Leptin inhibits lipogenesis, activates hepatic β-oxidation of fatty acids and can potentially reduce lipid deposition in the liver (Stern et al., 2016). Unlike adiponectin, leptin appears to have insulinotropic properties (Harris, 2014). Its increased concentrations are associated with insulin stimulation, cellular glucose uptake, and energy substrates (Steiner and Lang, 2017). Circulating leptin levels increase proportionally to the white fat depots' size and response to a meal (D'souza et al., 2017). However, fasting and cold temperature inhibit leptin action (Steiner & Lang, 2017). Therefore, alcohol's effect on leptin depends on the nutrition status (Park &

Ahima, 2015). Circulating leptin concentrations may increase, decrease or remain unchanged in rodents and humans under long-term alcohol intake (Szkudelski et al., 2004; Voican et al., 2015; Steiner & Lang, 2017). Generally, circulating leptin levels appear unrelated to alcohol intake but to feeding status and body weight (Stern et al., 2016; Steiner & Lang, 2017).

Alcohol consumption activates adipose tissue lipolysis that produces free fatty acids (FFA) in peripheral tissues and a decrease or no change in WAT (Blaner et al., 2017; Rasineni et al., 2019; Patel et al., 2022). However, circulating FFA concentration may not always reflect this change as the liver and the heart often mop them (FFA) up (Steiner & Lang, 2017), leading to the development of steatosis. Early research provides that stimulation of the β-adrenergic receptor signalling is a potent activator of lipolysis (Itaya, 1979; Yki-Järvinen et al., 1988). However, recent data suggest that the lipolysis rate rests significantly on the inhibitory action of insulin (Steiner & Lang, 2017). As alcohol increases lipolysis, its ability to dis-inhibit insulin must supersede its down-regulation of the β-adrenergic receptor signalling to boost lipolysis (Steiner & Lang, 2017).

2.6.3 Ethanol consumption: inflammation and oxidative stress

Dysregulated cytokine signalling, particularly cytokines released from macrophages, is common with chronic ethanol use (Meroni et al., 2019). Alcohol causes modification of the intestinal flora by mediating bacteria over-growth mucosal alteration and increased gut permeability (Meroni et al., 2019). Increased gut permeability leads to the release of lipopolysaccharide into the portal vein, which binds to Toll-like receptor 4, activating the innate immune system to mediate the release of pro-inflammatory cytokines IL-1α, IL-1β, TNFα and IL-6 as well as reactive oxygen species (ROS), which cause oxidative stress (Meroni et al., 2019). The role of TNF-α in AFLD remains uncertain, but it seems that it influences the effects

of *PPAR-α* and *SREBP-1c* (Kisseleva & Brenner, 2019). In rat models, *TNF-α* upregulates mRNA expressions of *SREBP-1c* in the liver, inducing the maturation of *SREBP-1c* protein (Tilg et al., 2016) and down-regulates the expression of *PPAR-α* (Beier et al., 1997). Chronic alcohol consumption in patients and animals elevates serum concentrations of *TNF-α* (Felver et al., 1990; Bird et al., 1990). In liver disease, IL-1β promotes the recruitment of inflammatory cells to the liver and activates hepatic stellate cells, contributing to the development of fibrotic tissue (Miura et al., 2010; Mehal & Imaeda, 2010). IL-1β can also induce triglyceride accumulation in hepatocytes (Miura et al., 2010; Petrasek et al., 2012) and its activities in synergy with TNF, mediate hepatocyte death (Miura et al., 2010; Petrasek et al., 2012; Szabo & Petrasek, 2015). Therefore, there is an increase in IL-1β in liver disease (Tilg et al., 2011). Similarly, IL-6 concentrations increase (Khoruts et al., 1991; Hill et al., 1992) but protect against ethanol-induced oxidative stress and mitochondrial dysfunction in hepatocytes via induction of metallothionein protein expression (El-Assal et al., 2004). **Figure 2.2** below shows that ethanol overconsumption downregulates *PPARα* hence inhibiting fatty acid oxidation. It increases homocysteine and ER stress responses leading to the activation of SREBP-1, a potent

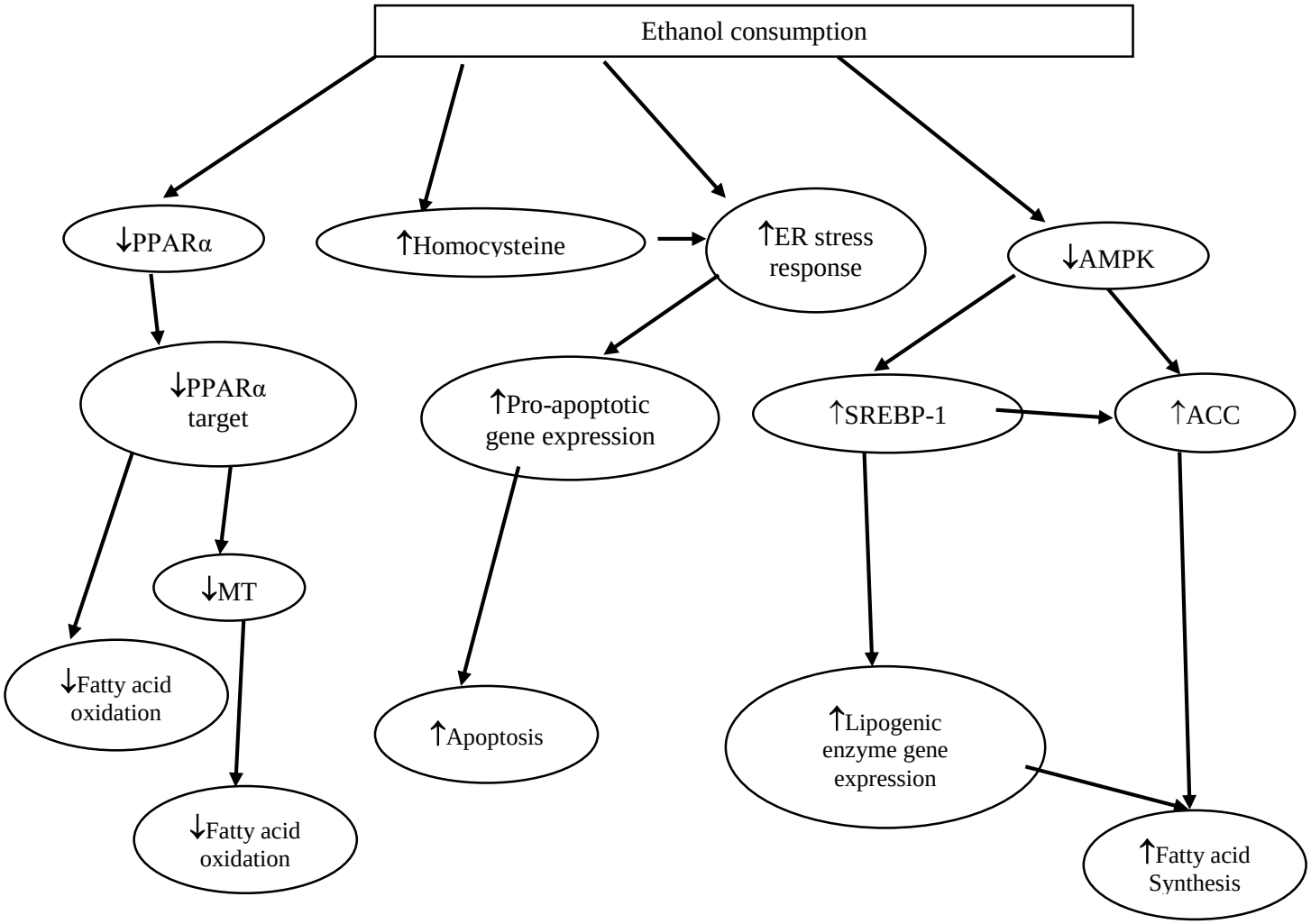

Figure 2.2: Effect of ethanol on transcriptional control genes. Source: Sozio and Crabb (2008)

inducer of lipogenesis. AMPK is also downregulated, causing increased fatty acid synthesis (Sozio and Crabb, 2008).

Chronic ethanol consumption activates CYP2E1. When CYP2E1 is activated, an unstable adduct gem-diol, which can break down into acetaldehyde, leads to NADPH oxidation, forming hydrogen peroxide (Zakhari, 2006). The formation of H_2O_2 leads to saturated fatty acid oxidation, lipid peroxidation, thus the appearance of lipid peroxides. Polyunsaturated fatty acids lose a hydrogen atom in a reaction with free radicals to form alkaloid radicals which can

enter into a reaction with oxygen to form peroxide radicals. These peroxides can induce oxidative injury, contributing to oxidative injury to liver tissue (Zhu et al., 2012).

2.7 Alcohol-induced liver disease

Despite extensive research and progress in liver diseases, millions of people are affected by acute or chronic liver conditions (Muriel, 2017). Globally, liver diseases affect more than 10% of the world's population and its mortal end-stage generally follows cirrhosis and liver cancer (Muriel, 2017). Varied factors characterise liver disease. Among liver diseases, non-alcoholic fatty liver disease ranks first, contributing 40% of liver diseases, while Hepatitis B virus, Hepatitis C virus and alcohol overconsumption contribute 30%, 15% and 11%, respectively (Muriel, 2017).

Alcohol-induced liver disease (ALD) is a term that describes disease manifestations of the liver that results from alcohol overconsumption. It includes a spectrum of injury to the liver ranging from steatosis to hepatitis with or without fibrosis (Gao & Bataller, 2011; Teschke, 2018). Altamirano and Bataller (2011) contend that the conditions underlying ALD range from fat deposition in the perivenular, mid-lobular and periportal hepatocytes, hepatic inflammation, necrosis, progressive fibrosis and superimposed hepatocellular carcinoma. Fatty liver, the first and earliest response to heavy drinking, develops in about 90% of heavy drinkers, with just about 8-20% of these individuals progressing to more severe forms of liver diseases, characteristically, advanced fibrosis and cirrhosis (Gao & Bataller, 2011; Ohashi et al., 2018). People with fatty liver disease usually present with early-mild steatosis which occurs in perivenular hepatocytes but in more severe cases, periportal hepatocytes get affected (Gao & Bataller, 2011). However some studies point out that the stages of the spectrum of alcohol-related liver injury do not necessarily evolve distinctly but may co-occur in an individual (Chacko & Reinus, 2016; R. Patel & Mueller, 2022). The distinct features of ALD include

mega-mitochondria, cholestasis, and hemosiderin deposition (Nakano & Fukusato, 2005). Histologically, alcoholic steatohepatitis and fibrosis are marked by neutrophil infiltration, hepatocyte ballooning, necrosis, the appearance of Mallory-Denk bodies, cholestatic changes, mega-mitochondria, as well as perivenular and pericellular fibrosis (Ohashi et al., 2018). Non-alcoholic fatty liver disease (NAFLD) has a similar pathological spectrum as ALD. However a mathematical model, the ALD/NAFLD index involving AST, ALT and mean corpuscular volume distinguishes ALD from NAFLD (Dunn et al., 2006). All other parameters, including the model above, have been criticised as not being precise in diagnosing ALD until a liver biopsy or imaging accompanies it, except for the history of excessive alcohol consumption (Carvalho Filho et al., 2007).

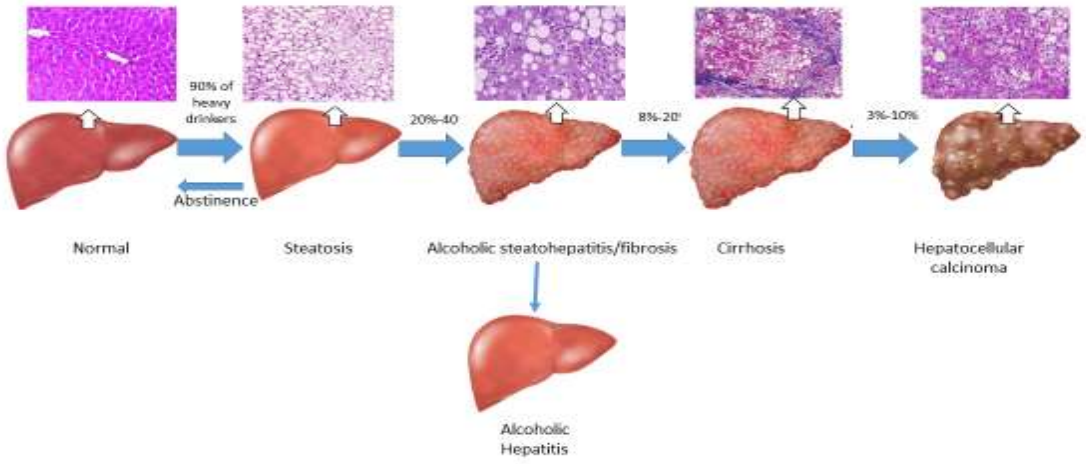

Figure 2.3: The developmental stages of alcoholic liver disease (Source: Ohasie et al., 2018).

Although controversy exists between the amounts of alcohol consumed and ALD development, studies indicate that the amount of alcohol consumed and the duration of consumption are

closely associated with cirrhosis (Singal & Anand, 2013). Most patients are likely to develop alcoholic hepatitis (AH) when they consume about 100 g/day of alcohol, equating to 6-7 drinks/day. However, patients who consume 30-50 g/d of alcohol for 5-10 years are at increased risk for AH (Singal & Anand, 2013). Bellentani and Tiribelli (2001) reported that the risk of developing liver damage steadily increases when ethanol consumption exceeds 30 g/day. Above the 30 g/day threshold, ALD and cirrhosis incidence correlate positively with increasing alcohol intake (Singal & Anand, 2013). Kamper-Jørgensen et al. (2004) indicated that the relationship between the quantity of alcohol ingested and liver disease development is not linear. About 90% of heavy drinkers develop steatosis, and 10 to 35% of patients with steatosis may progress to fibrosis, of which 8-20% will develop cirrhosis (Sorrell & Mukherjee, 1999).

The highest alcohol consumption per adult has been reported in Europe with Russians in the lead (Liangpunsakul et al., 2016). There is, however, little information regarding alcohol consumption among people from the Islamic Eastern Mediterranean and in the less developed Southeast Asia, particularly India (Liangpunsakul et al., 2016). In Africa and many other countries, alcohol consumption is generally declining but the harm of alcohol use is disproportionally high (Leslie et al., 2015). In South Africa, 60% of men attest to drinking and those that do, drink heavily with a *per capita* 30 litres of pure alcohol consumed annually (Leslie et al., 2015). Thirty-nine thousand deaths (6.4% of the total annual deaths) and 5% of disability-adjusted life years were attributable to alcohol among South Africans in 2012 (Leslie et al., 2015) demonstrating the heavy public health burden induced by alcohol.

A significant factor in the aetiology and pathogenesis of ALD is the type of alcohol consumed. One standard drink is a beverage containing 12-14 g of alcohol in 12 ounces (355ml, 4-5%

w/v) of beer, 5 ounces (125ml, 10-20% w/v) of wine and or 1.5 ounces (45ml, 40-50% w/v) of spirits (Guelinckx et al., 2011). Globally, the most commonly consumed alcohol is spirit (Basra, 2011). Heavy drinking involves the consumption of two or more drinks per day, while binge drinking is consuming five or more drinks for men within two hours and four or more drinks for women (Liangpunsakul et al., 2016). In a survey of more than 30,000 persons in Denmark, drinking beer or spirits was more likely associated with liver disease than drinking wine (Becker et al., 2002). According to Lu et al. (2004) alcohol drinking outside of meal times increases ALD risk by 2.7-fold than consuming alcohol at mealtimes only. Other related studies have also reported a strong association between five alcoholic drinks for men and four drinks for women in one sitting and the increased risk of ALD (Barrio et al., 2004). **Table 2.1** shows the quantity and duration of alcohol abuse and liver function.

Table 2. 1: Quantity and duration of alcohol abuse and liver function.

Liver function	Mean daily alcohol intake (mg of alcohol/kg of body weight) per hour	Average duration of alcohol abuse (years)
Normal liver function	90	7.7
Uncomplicated fatty liver	109	7.8
Severe steatofibrosis with inflammatory reactions	127	10.3
Chronic alcoholic hepatitis	125	11.9
Cirrhosis	147	17.1

Source: Mann et al. (2003).

2.8 Animals models for alcohol liver disease

Although not fully elucidated, the pathogenesis of ALD has been extensively studied (Abdelmegeed et al., 2017; Osna et al., 2017; Kong et al., 2019). However, advances in

treatment options have been slow because of a lack of viable and suitable animal models that mirror the full spectrum of ALD (Lamas-Paz et al., 2018). Rodents are used to study both acute and chronic phases of the condition. Differences exist between ALD animal models and what practically happens in humans (Arvola & Forsander, 1961; Lamas-Paz et al., 2018). Rodents have a strong aversion to alcohol (Arvola & Forsander, 1961). They may consume alcohol only for the calories and not as a craving (Lamas-Paz et al., 2018). These rodents catabolise alcohol faster than humans leading to relatively minor damage as may suit a researcher's requirement (Holmes et al., 1986). Importantly, their innate immune system is different from humans (Copeland et al., 2005) therefore, the degree of damage in rodents is far less than the researchers requirement.

Out of the several ALD animal models available, the most widely used include the Lieber-DeCarli liquid diet, ethanol *ad libitum* feeding, Tsukamoto-French intragastric infusion model, and the National Institute on Alcohol Abuse and Alcoholism model (Lamas-Paz et al., 2018). Except the Tsukamoto-French intragastric infusion model, the other three models could be employed to generate the early stages of ALD (Lamas-Paz et al., 2018). The ethanol *ad libitum* model, which is the simplest and easiest to use, mimics the typical drinking pattern in humans and importantly it causes relatively less animal mortality (Lamas-Paz et al., 2018). In this method, ethanol solution is administered to the rodents as the only source of drinking fluid, while rodents have free access to standard rat chow. The concentration of ethanol often used is 10-40% lasting for a duration of 8 - 70 weeks (Keegan et al., 1995; Brandon-Warner et al., 2012; Song et al., 2016). This method causes liver damage with clear steatosis and mild elevation of ALT and AST without advanced fibrosis and cirrhosis (Keegan et al., 1995; Brandon-Warner et al., 2012; Song et al., 2016). However, rodents' aversion to alcohol causes them to drink less, resulting in a low blood alcohol concentration (Best et al., 1949; Brandon-

Warner et al., 2012). Albeit, using ALD animal models and the DOHaD makes it feasible to elucidate the pathogenesis of ALD and create possible interventions for its management.

2.9 Treatment and potential therapies for ALD

The general management guidelines for ALD over the past five decade has included treatment of alcohol withdrawal, abstinence, nutritional support and pharmacological treatment of liver complications (Suk et al., 2014; Kim et al., 2016; Singh et al., 2017). Potential pharmacological targets include activation of antioxidant genes, inhibition of *CYP2E1* and the production of ROS, downregulation of inflammation through the activation of Kupffer cells and boosting of the innate immunity through the natural killer cells (Gao et al., 2009; Bertola et al., 2013; Altamirano et al., 2014; Luo et al., 2015; Buzzetti et al., 2017). Additionally, modulation of lipid regulatory genes play a considerable role in the management of ALD/AFLD (Kong et al., 2019).

Disulfiram is frequently prescribed to promote alcohol abstinence, however Gao & Bataller, (2011) indicated that it is not recommended in people with alcohol-related liver disease because of it causes hepatic toxicity. Glucocorticosteroids, anabolic-androgenic steroids, pentoxifylline, anti-tumour necrosis factor (infliximab and etanercept), colchicine, N-acetyl cysteine, propylthiouracil, vitamin E, antioxidants, S-adenosylmethionine, and milk thistle are some of the pharmacological agents used to treat ALD (Kong et al., 2019). Glucocorticosteroids, pentoxifylline, and anti-tumour necrosis factor suppress the synthesis of TNF-α to decrease inflammation (Singh et al., 2017). Pentoxifylline, by helping maintain normal renal function prevents hepato-renal syndrome (Singh et al., 2017). S-adenosylmethionine, N-acetyl cysteine, propylthiouracil, anti-oxidants including vitamin E and milk thistle are targeted at decreasing oxidative damage to liver cells (Suk et al., 2014).

Anabolic-androgenic steroids have been evaluated in alcohol-related liver disease because of their muscle building and direct effects on liver metabolism via decrease *de novo* lipogenesis in the liver (Kim et al., 2016; Balgoma et al., 2020). Colchicine has been evaluated in alcohol-related liver disease because of its anti-inflammatory and anti-fibrogenic properties by inhibiting collagen production (Suk et al., 2014; Kim et al., 2016; Singh et al., 2017). Novel pharmacological interventions such as anti-tumour necrosis factor (anti-TNF-α), granulocyte stimulation factor, rifaximin, and ursodeoxycholic acid, have also been used as a single therapy or in a combination with other interventions (Buzzetti et al., 2017). Despite this array of pharmacological therapies, none is ideal for the treatment of ALD.

2.10 Ethnomedicine in the management of alcohol liver disease

Literature shows that 34 extracts or individual compounds originated from medicinal plants such as *Cnidium monnieri*, Cusson (Apiaceae), *Curcuma longa* (Zingiberaceae) and *Pueraria lobata*, Ohwi (Leguminosae) are efficacious in the management of ALD and are in use in China (Ding et al. (2012). These plant-derived compounds and or extracts exhibit anti-oxidant and anti-inflammatory properties; they inhibit lipid synthesis but promote β-oxidation of fatty acids (Ding et al., 2012); properties that explain positive outcomes when they are used to manage ALD. Ethnomedicines, by virtue of their multiple health beneficial biological effects, are potential novel prophylactic and therapeutic agents against alcohol-induced liver injuries. However, there is a dire need to elucidate the underlying molecular mechanisms through which these ethnomedicines confer prophylactic and therapeutic effects against ALD and active ingredients involved (Madrigal-Santillán et al., 2014). Another challenge is that the chemical composition of herbal extracts might vary depending on plant origin and processing (Konieczynski et al., 2018). Therefore, in order to guarantee the consistency and repeatability of experimental results, standardized herbal extracts with consistent quality should be used and

their chemical characteristics profiled using modern analytical approaches, otherwise pure compounds extracted from these ethno-medicines may be used. One such pure compound is zingerone. The next section explores the therapeutic and prophylactic properties of zingerone against disease.

2.11 Zingerone

Zingiber officinale, commonly known as ginger, family Zingiberaceae, originates from South-East Asia and is a common spice and flavouring agent (Sharifi-Rad et al., 2017). Rarely, has ginger been found to cause allergic reactions when used as a spice to flavour food (Ahmad et al., 2015). In addition to containing significant micronutrients, ginger also known as zingibain, has several uses in the food industry (Unuofin et al., 2021). Ginger has many medicinal properties due to its varied phytochemistry (Sharifi-Rad et al., 2017) charactersied by many volatile and non-volatile compounds (Ahmad et al., 2015). Monoterpenoid hydrocarbons and sesquiterpene, the volatile constituents of ginger, account for its distinct taste and flavour of ginger and the non-volatile gingerols, shogaols, paradols and zingerone contribute to the medical aspect of ginger (Ahmad et al., 2015). Zingerone which makes up 9.25% of ginger is obtained directly by drying ginger or indirectly from the thermal degradation of gingerols or shogaols (Zhang et al., 2012).

2.11.1 Zingerone: chemical structure and biological activities

Zingerone is a member of the methoxy phenol family that is typified by a methyl compound attached to a benzene ring (Ahmad et al., 2015). Chemically, zingerone known as 4-(3-methoxy-4-hydroxyphenyl)-butane-2-one (vanillyl acetone) is a crystalline solid sparingly soluble in water and ether (Takizawa et al., 2012).

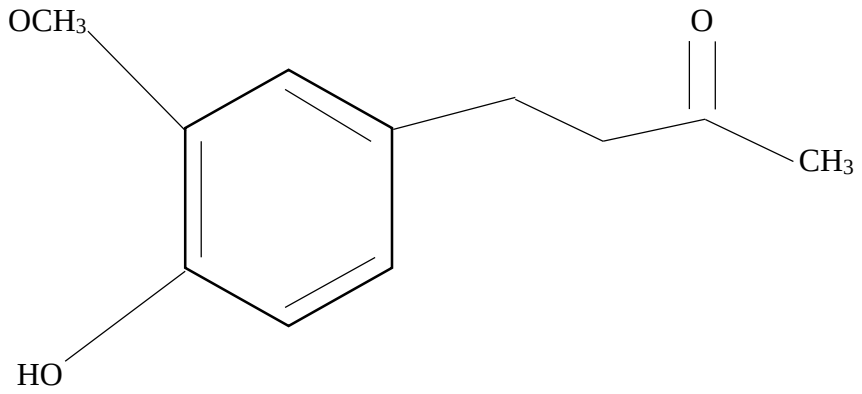

Figure 2. 4: Chemical structure of Zingerone; Source: Takizawa et al. (2012)

Zingerone exhibits antioxidant, anti-inflammatory, anti-cancer and anti-microbial properties that have a health beneficial effect (Takizawa et al., 2012; Ahmad et al., 2015). *In vivo*, zingerone is oxidised at its side chains which enhances solubility (Takizawa et al., 2012). Orally administered zingerone is eliminated from the body through its glucuronidation and sulfation in the liver (Ahmad et al., 2015).

2.11.2 Antioxidant properties

The anti-oxidant activity of zingerone against oxidative stress related conditions is widely documented. Orally administered zingerone (100 mg/kg/bwt) increased the activities of reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and reduced the peroxidative damage by suppressing *NF-κB* in rats with diabetes and nephron toxicity (Ahmad et al., 2018; Anwer et al., 2019; Cui et al., 2018; Kandemir et al., 2019). The suppression of *NF-κB* results in down-regulating downstream cytokines including IL1-β, IL-2, IL-6 and TNF-α. However, not all studies found zingerone to be a super antioxidant as some did not demonstrate increased anti-oxidant levels and/or did not demonstrate mediation of the prevention of lipid peroxidation (Kabuto et al., 2011; Eid et al., 2017). At 25 mg/kg body weight zingerone as a treatment decreased SOD and GPx activities in the liver demonstrating a failure to alleviate hepatic lipid peroxidation and oxidative stress

(Bashir et al., 2021). However in another study, a 7-day pre-treatment of rats with zingerone at 40 mg/kg body weight significantly increased GSH and SOD activities but had no effect on CAT activity against carrageenan-induced liver inflammation (Mehrzadi et al., 2021). Previously, Soliman and co-workers also showed that co-treatment of zingerone with whole body γ-irradiation at a single dose of 6 Gy in rats did not affect SOD levels (Soliman et al., 2018). Furthermore, eight weeks post-treatment with 75 mg/kg of zingerone against diabetic prostate complications in Wistar rats did not ameliorate oxidative stress (Eid et al., 2017). Kabuto et al. (2007), investigated this phenomenon of non-effective antioxidant potential of zingerone in 6-hydroxydopamine-induced parkinson's disease in rats and concluded that pre-treatment with zingerone was more effective than post-treatment or co-treatment against oxidative stress conditions. The study posited that post and/or onset treatment with zingerone is not effective because during the outset of a disease condition, the rate at which ROS is generated overwhelms the ROS quenching effect of zingerone hence its ineffectiveness (Kabuto et al., 2007; Kabuto & Yamanushi, 2011). This suggest that the period of zingerone intake is critical to its potency as an anti-oxidant. However, Mir et al. (2018) showed that both pre-treatment and co-treatment against cyclophomide-induce hepatic toxicity works effectively, despite the relatively higher concentration of antioxidant biomarkers in the pre-treated compared to the co-treated group.

2.11.3 Cardio-metabolic protective properties

Cardiometabolic diseases and their associated metabolic derangements such as dyslipidemia, obesity and insulin resistance, place a heavy burden on the healthcare system and the patients' quality of life (Benjamin et al., 2019). While conventional pharmacological agents and lifestyle adjustments are tools used to manage cardiometablic diseases, side effects, poor access, and lack compliance to the lifestyle adjustments results in poor outcomes (Barteková et al., 2021). Pre-treatment of with zingerone at 25 mg/kg body mass for three weeks mitigated against

cistaplastin-induced or γ-irradiation-induced cardiac abnormalities by increasing lactacte dehydrogenase and creatine kinase myocardial band (MB) activities and the concentrations of cardiac troponin T and B-natriuretic peptides (Soliman et al., 2018). Muhammed et al. 2021 demonstrated that neonatal orally administered zingerone mediated cardiometabolic protection in adulthood by lowering lipids in male and female rats and significantly decreased the terminal body mass of female rats. In rats, co-treatment with zingerone was also shown to effectively decrease plasma triglyceride and total cholesterol concentration and increase high density cholesterol (Anwer et al., 2019; Cui et al., 2018). Furthermore, in high-fat diet fed and diabetic rats orally administered zingerone decreased glucose, insulin, c-peptide and glycosylated haemoglobin A1c (Narayanan & Jesudoss, 2016; Ahmad et al., 2018; Cui et al., 2018; Mohammed, 2022). Wei et al. (2017) report 6-paraol as the active component of ginger that mediated anti-hyperglycaemic activity in high-fat diet fed rats. Some studies also reported that zingerone has no significant effect on hyperglycaemia (El-Bassossy et al., 2017; Muhammad et al., 2021a). However, the cardiometabolic protective effects of zingerone are posited to emanate from its anti-oxidant and lipolytic activities and ability to activate the AMPK signalling pathways (Han et al., 2008; Anwer et al., 2019; Mohammed, 2022).

2.11.4 Hepatoprotective properties

Rodent studies show that zingerone has hepato-protective properties. In p-aeroginosa LP-induced liver injury, orally administered zingerone downregulated mRNA expression of *TLR4, TNF-α* and *inducible nitrogen oxide synthase (iNOS)* and in BALB/c mice it decreased serum endotoxin and surrogate markers of liver function (Kumar et al., 2014). Mani et al. (2016) showed that in experimental rats, supplementation of ALD pharmacotherapy with zingerone reversed the ethanol-induced liver disease pointing to its therapeutic potential against ethanol-induced hepatotoxicity. Zingerone is reported to protect against λ-mediated infra-red-induced

hepatotoxicity in rats by upregulating MAPK signal transduction, decreasing inflammatory markers and and NADPH oxidase activity (Mohamed & Badawy, 2019). In fructose-induced non-alcoholic fatty rats, the administration of zingerone at 100 mg/kgbwt and 40 mg/kgbwt reversed the dietary fructose-induced hepatic histopathological alterations (Narayanan & Jesudoss, 2016; Muhammad et al., 2021b). Cheong et al. (2016) demonstrated that zingerone ameliorated the effect of carbon tetrachloride- and dimethylnitrosamine-induced liver injuries in rats. Interestingly, in liver cancer, reconstituted zingerone nanoparticles showed protective and therapeutic effects by significantly inhibiting the activity of protein kinase B (AKT) and the expression of nuclear factor-kappa beta (*NF-KB*) and by activating the caspase signalling pathway which mediates anti-proliferation and anti-tumourigenic activities (Kung et al., 2019). These zingerone nanoparticles induced effects that reversed cell cycle progression and DNA damage, but induced apoptosis in cancer cells (Kung et al., 2019). Thus by mediating decreased production of inflammatory markers and NADPH activity, upregulated MARK pathway and enhancing maintenance of liver cell integrity, zingerone is hepatoprotective.

2.11.5 Anti-inflammatory properties

Endotoxin or lipopolysaccharide plays is a key factor in alcohol-induced liver damage via induction of pro-inflammatory cytokines (Slevin et al., 2020). In male C57BL/6 mice 0.72 mg/kg i.v administration of zingerone reversed LPS-induced liver failure by decreasing toll-like receptor 4 (TLR4) and MyD88 protein expression (Lee et al., 2018) which consequently resulted in reduced serum TNF α and IL-6; biomarkers of inflammation (Lee et al., 2018). In adjuvant-induced rheumatoid arthritis rats, the use of zingerone (25 mg/kg body weight) from onset of the disease condition ameliorated the severity of inflammation in the joint (Bashir et al., 2021). Bashir et al. 2021 concluded that zingerone's anti-inflammatory activity is due to its ability to downregulate *NF-Kβ*, that normally initiates and intensifies inflammation. Although

other signalling pathways have been proposed to be the action mechanism of zingerone's anti-inflammatory, the *NF-Kβ* stands out (Kumar et al., 2014; Hsiang et al., 2015; Ahmad et al., 2018; Rehman et al., 2019; Kucukler et al., 2020). Zingerone's antioxidant, cardiometabolic and hepatoprotective and anti-inflammatory properties makes it a feasible intervention that can be administered in early-life to programme for protection against metabolic consequences of neonatal and adult exposure to alcohol. However, no data exist on its potential to programme against metabolic effect and alcohol-induced liver disease associated with alcohol consumption in adulthood.

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CHAPTER THREE: ORALLY ADMINISTERED ZINGERONE DOES NOT MITIGATE ALCOHOL-INDUCED HEPATIC OXIDATIVE STRESS IN GROWING SPRAGUE-DAWLEY RAT PUPS

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3.0 Abstract

Neonatal alcohol exposure (NAE) can induce oxidative stress. We determined whether zingerone (ZO), a phytochemical with anti-oxidant activity, can mitigate the negative impact of neonatal alcohol-induced oxidative stress. Seventy ten-day-old Sprague-Dawley rat pups (35 males, 35 females) were randomly assigned and administered the following treatment regimens daily from postnatal day (PND) 12-21: group 1 - nutritive milk (NM), group 2 – NM +1g/kg ethanol (Eth), group 3 - NM + 40 mg/kg ZO, group 4 – NM + Eth+ ZO. Growth performance, blood glucose and plasma triglycerides (TGs), total cholesterol, HDL-cholesterol, leptin and insulin concentration were determined. Cytochrome p450E21(CYP2E1) and thiobarbituric acid (TBARS); markers of hepatic oxidative stress and catalase, superoxide dismutase (SOD) and total glutathione (GSH), anti-oxidant markers of the pups were determined. Oral administration of ethanol (NM+Eth), zingerone (NM+ZO) and combined ethanol and zingerone (NM+Eth+ZO) did not affect growth performance and insulin and leptin concentration of the rats ($p > 0.05$). Ethanol significantly reduced plasma TGs concentration of female rats ($p = 0.04$ vs control). However, ethanol and/or its combination with zingerone decreased hepatic GSH ($p = 0.02$ vs control) and increased CYP2E1 ($p = 0.0002$ vs control) activity in male rat pups. Zingerone had no effect ($p > 0.05$ vs control) on the rats' CYP2E1, GSH, SOD and catalase activities. Neonatal alcohol administration elicited hepatic oxidative

stress in male rat pups only, showing sexual dimorphism. Zingerone (NM+ZO) prevented an increase in CYP2E1 activity and a decrease in GSH concentration but did not prevent the alcohol-induced hepatic oxidative stress in the male rat pups.

Keywords: zingerone, alcohol, oxidative stress, rat pups

3.1 Introduction

Alcohol consumption during pregnancy affects the mother and the developing foetus (Caputo et al., 2016). The umbrella term, foetal alcohol syndrome disorders (FASD), encompasses foetal alcohol syndrome (FAS), partial FAS, alcohol-related neurodevelopmental disorders and alcohol-related congenital disabilities (Caputo et al., 2016; Lange et al., 2017). Foetal alcohol syndrome is the most severe form of FASD (Caputo et al., 2016). In the general population, among every 13 pregnant women who consume alcohol, one is likely to have a child with FASD (Lange et al., 2017). FASD is avoidable if pregnant women abstain from excessive alcohol consumption. Studies indicate that women who consume alcohol during pregnancy are likely to continue indulging in the habit during lactation (Tran et al., 2015). Although alcohol consumption during breastfeeding may not directly cause FASD, it can have detrimental effects on the child and possibly impact health later in life (Anderson, 2018).

Breastmilk is nutritional for babies such that exclusive breastfeeding is recommended for the first six months; after that, solid foods can supplement breastfeeding until the infant is two years old (WHO, 2009). Studies have shown that alcohol can quickly move into breastmilk via passive diffusion (D'Apolito, 2013). Alcohol reaches the breastmilk 0.5-1hr after the mother has consumed it (Mennella, 1998). About 95% of the mother's consumed dosage gets to her blood, and this closely parallels the amount of alcohol that enters the breastmilk (Anderson, 2018a). However, only 5-6% of the maternal blood alcohol level gets to the breastfeeding infants (Mennella, 1998). Unfortunately, in newborns, the liver has not developed the capacity

to metabolize and detoxify alcohol, thus increasing the chances of oxidative stress (Lavoie & Tremblay, 2018). Several studies identify oxidative stress as the pre-eminent factor in ethanol teratogenicity (Bhatia et al., 2019). Three major pathways are involved in ethanol-induced oxidative stress, namely mitochondrial electron transport chain malfunction and increased activities of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the cytochrome P450 subfamily 2E1 (CYPE21) (Bhatia et al., 2019). These pathways generate reactive oxygen species, which, when in excess, lead to toxic effects such as increased lipid peroxidation, inactivation of enzymes, induction of DNA mutations and destruction of cell membranes (Bhatia et al., 2019). Excessive generation of reactive oxygen species overwhelms an organism's anti-oxidant system, leading to oxidative stress and organ damage (Varga et al., 2017). Alcohol's effects on the developing foetus is a major area for research focus (Caputo et al., 2016). However, data on the effects of maternal alcohol consumption on offspring during lactation is scarce. It is important to note that the lactation period can have independent but equally significant impacts on metabolic programming (Ellsworth et al., 2018).

Several studies have shown that the administration of phytochemicals with anti-oxidant activity during the suckling period can protect against oxidative stress-related conditions (Lembede et al., 2018; Nyakudya et al., 2018). Zingerone, a phytochemical derived mainly from ginger (Ahmad et al., 2015), possesses anti-hyperlipidemic, anti-diabetic, anti-oxidant and anti-inflammatory properties (Ahmad et al., 2015, Ahmad et al., 2018; Cui et al., 2018). Based on the aforementioned pharmacological properties of zingerone, we determined whether zingerone can protect against alcohol-induced oxidative stress and metabolic effects in rat pups.

3.2 Materials and methods

3.2.1 Study site and ethics

All protocols and experimental procedures were approved by the Animal Research Ethics Committee of the University of Witwatersrand (Ethical clearance number: 2019/10/57/B). The study complied with accepted laboratory animal use and care principles stipulated in the South African National Standard (SANS 10386:2008) and the Animals Protection Act, 1962: Act No. 71. The study was undertaken at the Wits Research Animal Facility (WRAF).

3.2.2 Animal housing

This experiment used seventy (35 males and 35 females) 10-day-old Sprague Dawley rat pups obtained from 7 dams (litter 8-12). The pups were allowed to nurse freely from their respective dams throughout the suckling period from postnatal day (PND) 1-21. Dam and litters were kept in a temperature-controlled room (24±2°C) with a 12/12 h light-dark cycle with the light on from 7:00 h to 19:00 h. The animals were housed in acrylic cages with wood shavings for bedding. The wood shavings were changed twice a week.

3.2.3 Study design

Rat pups were assigned into four groups as follows: group 1-nutritional milk (NM), group 2 - NM+1 g/kg of alcohol (Eth), group 3 – NM+40 mg/kg of zingerone (ZO), and group 4 – NM+ 1g/kg of alcohol (Eth) + 40mg/kg of zingerone (ZO). Nutritive milk [Kitty milk (V 16752 Act 36/1947), Kyron Labs; Johannesburg, South Africa] was used as a vehicle for alcohol and zingerone. Supplementary data (S3.1-S3.6) provides a comparative analysis between using water or nutritive milk as control. Nutritive milk had no considerable effect on the rats' organ morphometry and metabolic effects; thus, we used the nutritive as a control. After two days of

habituation, the pups were administered via orogastric gavage their respective treatment regimens at a dose of 10 mL/kg between 9:00 am-11:00 am daily from postnatal (PND) 12-21. PND10-21 coincides with the neonatal suckling period, a critical window of developmental plasticity in rats (Picut et al., 2015). Zingerone [Sigma-Aldrich (W312401); Johannesburg, South Africa] dosage (40mg/kg) used in the current study is similar to a previously described study by Muhammad et al. (2021). The alcohol (ACE; Johannesburg, South Africa) concentration (1g/kg) used in this study parallels maternal blood alcohol and does not cause neurological effects (West et al., 1989; Anderson, 2018). The alcohol concentration was estimated based on body mass and the human to rat dose conversion ratio (human: rat) of 1:6.17 as previously done (Reagan-Shaw et al., 2008).

3.2.4 Terminal procedures, sample collection and processing

On PND 22, the terminal body masses of the rat pups were measured using a calibrated electronic scale (Snowrex, Johannesburg, South Africa). After that, the rat pups were euthanized using an overdose (150 mg/kg) of sodium pentobarbital (Eutha-naze®, Bayer, Johannesburg, South Africa). Before the intraperitoneal injection of euthanaze, a drop of blood drawn via pinprick on the tail vein was used to determine blood glucose with a calibrated blood glucose meter (Contour Plus Bayer®, Johannesburg, South Africa). The blood was drawn via cardiac puncture with 21 G needle into heparinized tubes and then centrifuged at 3000 × *g* for 15 mins to harvest plasma which was then stored at -80˚C till use for biochemical assays. Following blood collection, the liver and pancreas were dissected out and weighed using an electronic balance (Snowrex, Johannesburg, South Africa). Each liver was then rinsed in cold saline, and then divided into two samples and then frozen stored at -20˚C for the determination of liver triglycerides and anti-oxidants assays.

After harvesting plasma, the right hind-limb tibia of each rat was dissected from the carcass and de-fleshed. The de-fleshed tibiae were then dried to a constant mass in an oven (Salvis ®, Salvis Lab, Switzerland) at 50˚C for six days. The dry mass of the tibiae was measured with an electronic scale (Presica 310M, Presica Instruments AG, City, Switzerland) and the length with digital callipers (KTV 150 digital calliper, Elandsfontein, South Africa). The mass to length ratio of the tibiae was computed using the formula given by Seedor et al. (1991) : tibiae density (mg/mm) = dry mass of bone (mg) /bone length (mm).

3.2.5 Liver tissue homogenization

Ten per cent of the liver sample was minced and homogenised in phosphate buffer (0.1 M, pH=7.4) with an ultra turrax homogeniser (T-25 basic, Janke & Kunkel Ultra Turrax, Germany). The resultant homogenate was centrifuged at 3000 × *g* for 60 mins at 4˚C. The harvested supernatant was used to determine hepatic oxidative stress, anti-oxidant enzyme activities and triglyceride content.

3.2.6 Determination of hepatic CYP2E1 concentration

Rat-specific CYP2E1 ELISA kits (Elabscience ®, Wuhan, Hubei Province, China) were used to determine the hepatic CYP2E1 concentration (sensitivity range: 3.13-200 ng/mL). The test employed a sandwich ELISA principle. The assay was performed following the manufacturer's instructions. The optical density of the resulting reaction was measured at 450 nm on a microplate reader (Thermo Fisher Scientific Inc, Finland), and the sample concentrations were extrapolated from the standard curve.

3.2.7 Determination of hepatic thiobarbituric acid

Thiobarbituric acid (TBARS) concentration in the liver homogenate was estimated by the method of Niehaus & Samuelsson (1968). Briefly, 0.5 mL of the liver tissue homogenate sample was diluted with 0.5 mL of distilled water, after which 2.0 mL of the working reagent (TBA-TCA-HCl in a ratio of 1:1:1) was added. The mixture was boiled for 15 mins and allowed to cool on ice for 5mins. It was then centrifuged, and the supernatant was obtained. The absorbance of the supernatant was read spectrophotometrically (Beckman coulter, USA, California) at 532 nm.

3.2.8 Determination of catalase activity

Liver catalase was estimated using the method described by Sinha (1972). To 0.1 mL of liver tissue homogenate in a glass test tube, 0.90 mL of phosphate buffer and 0.4 mL of H_2O_2 were added. The reaction was arrested at 15, 30, 45 and 60 seconds with 2 mL of dichromate acetic acid solution. The reaction was stopped by immersing the test tubes containing the reactants in a water bath with boiling water for 10 mins and cooled on ice. The color developed was measured on a spectrophotometer (Beckman coulter, USA, California) at 610 nm. Catalase activity was expressed as µmoL of H_2O_2 consumed/min mg of protein for tissue.

3.2.9 Determination of superoxide dismutase activity and glutathione protein concentration

Hepatic superoxide dismutase (SOD) activity and total glutathione (GSH) protein were determined with biochemical assays kits (Elabscience®, Rat ELISA kit, Wuhan, Hubei Province, China) following the manufacturer's instructions. SOD activity was measured by the water-olubletetrazoliumsalt (WST-1) method. Xanthine oxidase (XO) catalyzes WST-1 and reacts with O^{2-} to generate a water-soluble formazan dye. Hepatic SOD inhibits the

disproportionation of superoxide anions; thus, there is a negative correlation with the amount of formazan dye formed. SOD activity was determined by the colorimetric analysis of WST-1 products developed at 450 nm on a microplate reader (Thermo Fisher Scientific Inc, City, Finland). Total protein was estimated by the Bradford method (Bradford, 1976). Briefly, 5 µL of the liver homogenate was added to 250 µL of Sigma's Bradford reagent (B6916; Sigma, Germany). The reactants were mixed thoroughly and incubated for 10mins at room temperature. The plate was read at 595 nm on a microplate reader (Thermo Fisher Scientific Inc, Finland). For the determination of total GSH concentration, oxidized glutathione is reduced by glutathione reductase in the sample, and the reduced GSH react with 5,5'-dithio-bis (2-nitrobenzoic acid) to produce oxidized glutathione (GSSG) and yellow 2-nitro-5-mercapto-benzoic acid (TNB). The amount of total glutathione determines the amount of yellow 2-nitro-5-mercapto-benzoic acid (TNB) formed. This was measured colorimetrically at a wavelength of 412 nm on a microplate reader (Thermo Fisher Scientific Inc, City, Finland).

3.2.10 Determination of liver triglyceride content

With the liver homogenate obtained, triglyceride (TG) concentration was assayed using kits from Elabscience ®, Wuhan, Hubei Province, China) following the manufacturers' instructions. In summary, 2.5 µL of liver homogenate was diluted with 2.5 µL of distilled water and mixed with 250 µL of TG reagent. The mixture was incubated at 37°C for 10mins, and the absorbance was read on a microplate reader (Thermo Fisher Scientific Inc, City, Finland) at 510nm. Hepatic TG was expressed as gprot/L.

3.2.11 Determination of plasma lipid profile

Plasma triglyceride, total cholesterol and high-density lipoprotein cholesterol concentration were determined using colorimetric assay kits as per the manufacturer's instruction

(Elabscience ®, Wuhan, Hubei Province, China). Low-density lipoprotein cholesterol concentration was estimated using the Friedewald equation (Knopfholz et al., 2014):

$$\text{LDL} - \text{Cholesterol} \left(\frac{\text{mmol}}{\text{L}}\right) = \text{Total cholesterol} - \left(\text{HDL} - \text{choleterol} + \frac{\text{Triglycerides}}{2.2}\right)$$

3.2.12 Determination of plasma leptin and insulin concentration

Rat-specific insulin (INS), leptin (LEP) ELISA kits (Elabscience®, Rat INS (Insulin) ELISA kit, Wuhan, Hubei Province, China) were used to determine the plasma insulin and leptin concentration, respectively, according to the manufacturer's instructions. Insulin resistance was computed based on the homeostasis model assessment of insulin resistance (Matthews et al., 1985):

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

3.3 Data Analysis

GraphPad Prism 8 software was used to analyse the data. Data were expressed as mean ± standard deviation. A one-way ANOVA was used followed by the multiple-comparisons Tukey *post hoc* test to compare the means. Statistical significance was considered when $p < 0.050$.

3.4 Results

3.4.1 Effects on growth performance

Figure 3.1 shows the effect of the treatment regimens on the body mass of the rat pups over the experimental period. There was no statistically significant difference in the induction [p = 0.925 (female); p = 0.704 (males); Figure 3.1] and terminal [p = 0.772 (females); p = 0.148 (males);

Figure 3.1] body masses of the rat pups across treatment groups in female and male rat pups. However, all rat pups grew significantly ($p < 0.0001$; Figure 3.1).

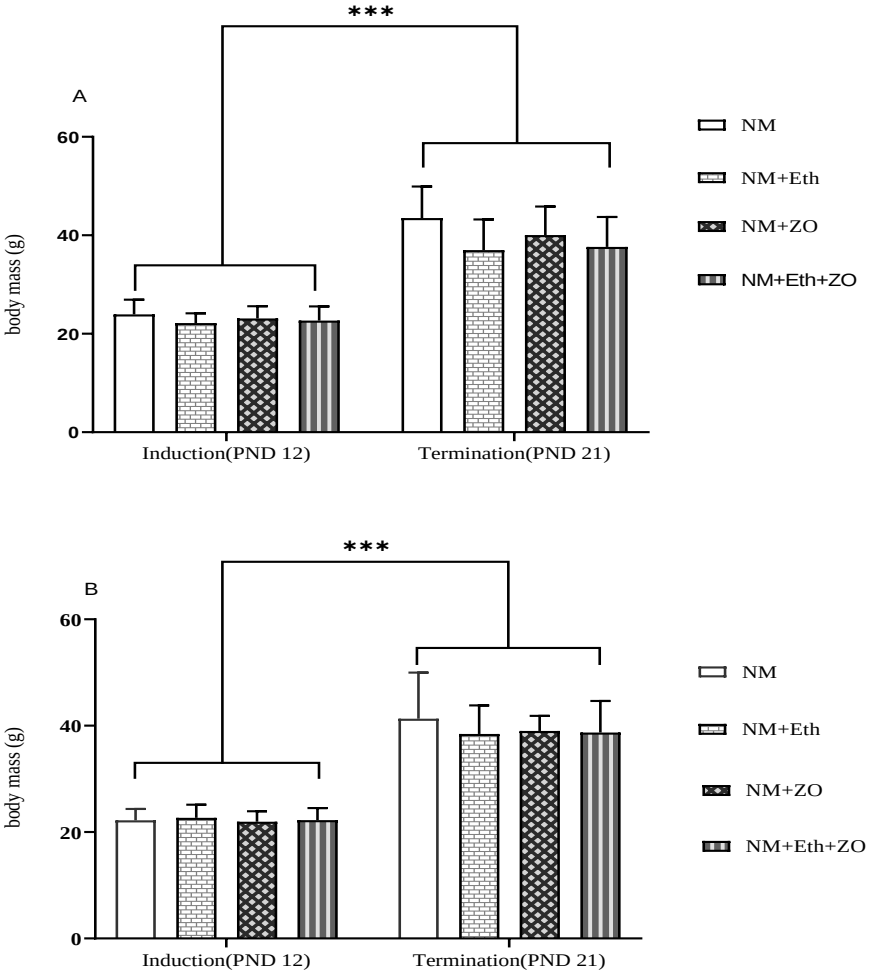

Figure 3.1: Effect of neonatal oral administration of zingerone on the terminal body masses of male (A) and female (B) rat pups exposed to alcohol

Data presented as mean ± SD. ***=$p < 0.050$ when induction body mass is compared to terminal body mass. NM=gavaged with 10 mL/kg of nutritive milk: NM+Eth=gavaged with 1 g/kg bwt of alcohol in nutritive milk; NM+ZO = gavaged with 40 mg/kg bwt of zingerone in nutritive milk; NM+Eth+ZO=gavaged with 1 g/kg bwt of alcohol + 40 mg/kg bwt of zingerone in nutritive milk. n = 8-10

The empty carcass of the females was similar across treatment groups ($p > 0.539$; Table 3.1). Similarly, there was no significant difference in the empty carcass of the male rat pups ($p = 0.144$; Table 3.1). Neonatal exposure to alcohol, zingerone and or a combination of alcohol and zingerone had no effect on the tibiae mass, length and mass/length ratio of the male and female rat pups ($p > 0.05$; Table 3.1).

Table 3.1: The effect of neonatal oral administration of zingerone on long bone indices and empty carcass in suckling pups exposed to alcohol

Parameter	Sex	NM	NM+Eth	NM+ZO	NM+Eth+ZO
Tibia mass (mg)	Female	46.0±13.3	40.6±6.4	42.3±4.5	41.1±6.6
	Male	48.9±9.8	41.6±5.3	43.0±5.2	41.8±5.3
Tibia length(mm)	Female	15.4±1.2	15.2±0.7	15.3±0.6	15.1±0.6
	Male	15.9±1.3	14.9±1.1	15.0±0.8	14.9±0.5
Tibia mass/length(mg/mm)	Female	2.9±0.6	2.7±0.3	2.8±0.2	2.7±0.3
	Male	3.1±0.4	2.8±0.3	2.86±0.20	2.8±0.3
Empty carcass	Female	32.8±7.1	30.2±4.1	30.2±3.8	29.9±4.6
	Male	33.5±5.4	28.7±4.6	30.8±4.3	28.3±5.5

Data presented as mean ± SD. $p > 0.050$. NM=gavaged with 10 mL/kg bwt of nutritive milk: NM+Eth=gavaged with 1 g/kg bwt of alcohol in nutritive milk; NM+ZO=gavaged with 40 mg/kg bwt in nutritive milk; NM+Eth+ZO = gavaged with 1 g/kg bwt of alcohol + 40 mg/kg bwt of zingerone in nutritive milk. n = 8-10 per treatment

3.4.2 Effects on gross liver and pancreas morphometry

Absolute and relative liver masses were similar across the treatment groups for males and females ($p > 0.05$; Table 3.2). The absolute pancreas mass was significantly different in male rat pups ($p = 0.012$; Table 3.2): alcohol (NM+Eth) significantly reduced the absolute pancreas mass ($p = 0.010$ vs control), but pancreata mass relative to tibia length was similar in the male and female rat pups across treatment regimens ($p > 0.05$; Table 3.2).

Table 3.2: The effect of neonatal oral administration of zingerone on liver and pancreas weight in suckling rat pups exposed to alcohol

Parameter	Sex	NM	NM+Eth	NM+ZO	NM+Eth+ZO
Liver mass (g)	Female	1.80±0.37[a]	1.83±0.23[a]	1.81±0.30[a]	1.87±0.23[a]
	Male	1.19±0.21[a]	1.23±0.14[a]	1.91±0.21[a]	1.26±0.24[a]
Liver rTL(g/mm)	Female	1.93±0.40[a]	1.70±0.35[a]	1.83±0.31[a]	1.26±0.37[a]
	Male	1.18±0.15[a]	1.11±0.07[a]	1.22±0.19[a]	1.10±0.18[a]
Pancreas (g)	Female	0.21±0.04[a]	0.21 ±0.06[a]	0.22±0.08[a]	0.20±0.06[a]
	Male	0.24±0.05[a]	0.12±0.04[b]	0.21±0.06[ab]	0.18±0.04[ab]
PancreasrTL(g/mm)	Female	0.14±0.03[a]	0.14±0.04a	0.15±0.04[a]	0.12±0.05[a]
	Male	0.13±0.06[a]	0.10±0.03[a]	0.14±0.03[a]	0.14±0.04[a]

Data presented as mean ± standard deviation. p-value was set at $p < 0.050$. [ab] = within row means with different letters significantly different at $p < 0.050$. NM = gavaged with 10 mL/kg bwt of nutritive milk; NM+Eth = gavaged with 1 g/kg bwt of alcohol in nutritive milk; NM+ZO = gavaged with 40 mg/kg bwt of zingerone in nutritive milk; NM+Eth+ZO = gavaged with 1 g/kg bwt of alcohol + 40 mg/kg bwt of zingerone in nutritive milk. Liver rTL= liver mass

relative to tibial length, PancreasrTL= pancreas mass relative to tibial length. n=8-10 per treatment

3.4.3 Effects on oxidative stress and anti-oxidants

In the females, there was no statistical significance across treatment for oxidative stress biomarkers; CYP2E1 ($p = 0.126$; Fig 3.2A) and TBARS ($p = 0.946$; Fig 3.3D). Similarly, treatments had no effect on the anti-oxidants catalase ($p = 0.934$; Fig 3.3A), SOD ($p = 0.446$; 3.3B) and GSH ($p = 0.074$; Fig 3.3C) in the female rat pups. In male rat pups, alcohol (NM + Eth) significantly increased hepatic CYP2E1 concentration ($p = 0.0002$ vs control; Figure 3.4A). The administration of zingerone (NM + ZO) resulted in similar CYP2E1 concentration ($p = 0.188$; Figure 3.4A) with that of rats administered the control (NM). Co-administration of Eth and ZO increased ($p = 0.047$ vs control) CYP2E1 concentration. Catalase and SOD activity were similar in the rat pups across treatment regimens ($p > 0.05$; Figure 3.5A&3.5B). Neonatal oral administration of alcohol (NM+Eth) significantly decreased ($p = 0.041$ vs control; Figure 3.5C) GSH concentration. However, GSH concentration was significantly higher in the ZO group (NM+ZO) compared to ethanol group (NM+Eth) ($p = 0.003$; Figure 3.5C), but the ZO did not prevent the alcohol-induced decrease in combined alcohol and ZO (NM+Eth+ZO) group ($p = 0.717$ vs control). There was no difference in GSH concentration between the ethanol group (NM+Eth) and the combined ethanol and ZO (NM+Eth+ZO) group ($p = 0.619$). Treatment regimens had no effects on the TBARS concentration of the male rat pups ($p=0.371$; Figure 3.5D).

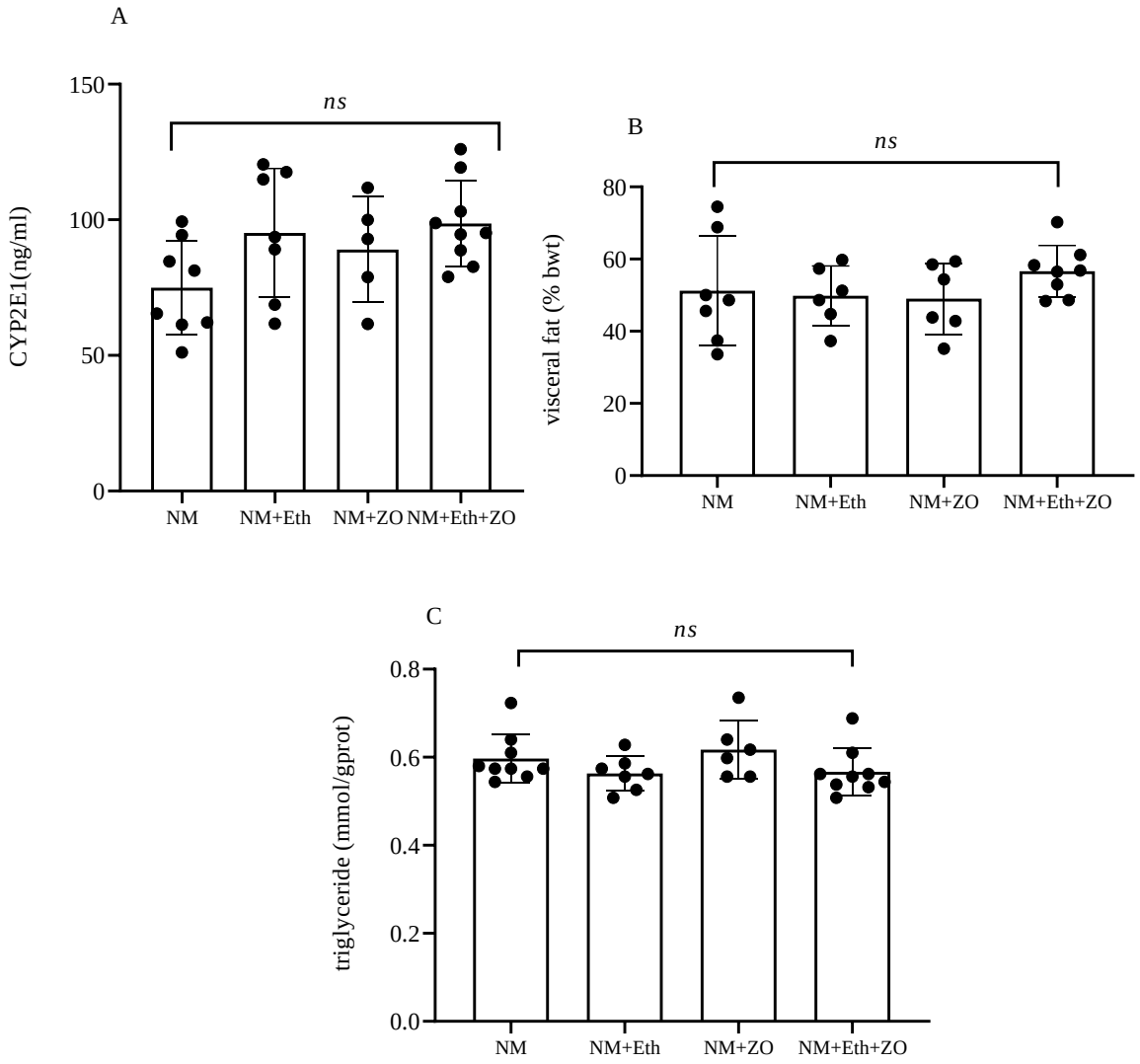

Figure 3.2: **Effect of neonatal oral administration of zingerone on (A) liver cytochrome p450, (B) visceral fat mass (% bwt), (C) and triglycerides of female rat pups exposed to alcohol**

Data presented as mean ± SD. ns = p > 0.050 when statistical comparison is non-significant across treatment. NM = gavaged with 10 mL/kg of nutritive milk: NM+Eth = gavaged with 1 g/kg bwt of alcohol in nutritive milk; NM+ZO = gavaged with 40 mg/kg bwt of zingerone in nutritive milk; NM+Eth+ZO=gavaged with 1 g/kg bwt of alcohol + 40 mg/kg of zingerone in nutritive milk. n=5-8 per treatment

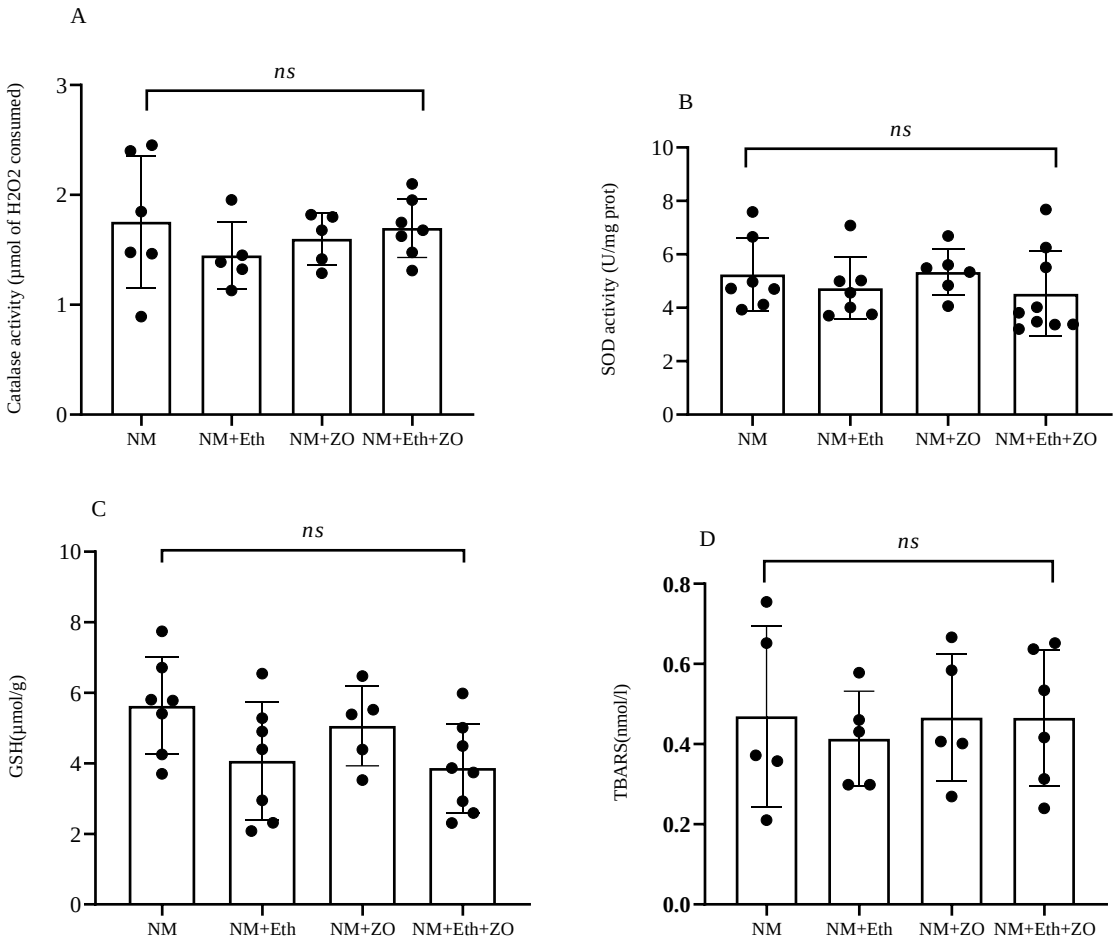

Figure 3.3: Effect of neonatal oral administration of zingerone on anti-oxidant biomarkers (A) catalase activity (B) superoxide dismutase activity (C) glutathione proteins and oxidative stress marker (D) TBARS in female rat pups exposed to alcohol.

Data are presented as mean ± SD. ns = $p > 0.05$ when statistical comparison is non-significant across treatment. NM = gavaged with 10 mL/kg of nutritive milk: NM+Eth = gavaged with 1 g/kg bwt of alcohol in nutritive milk; NM+ZO = gavaged with 40 mg/kg of zingerone in nutritive milk; NM+Eth+ZO = gavaged with 1 g/kg bwt of alcohol + 40 mg/kg of zingerone in nutritive milk. n=5-8 per treatment group.

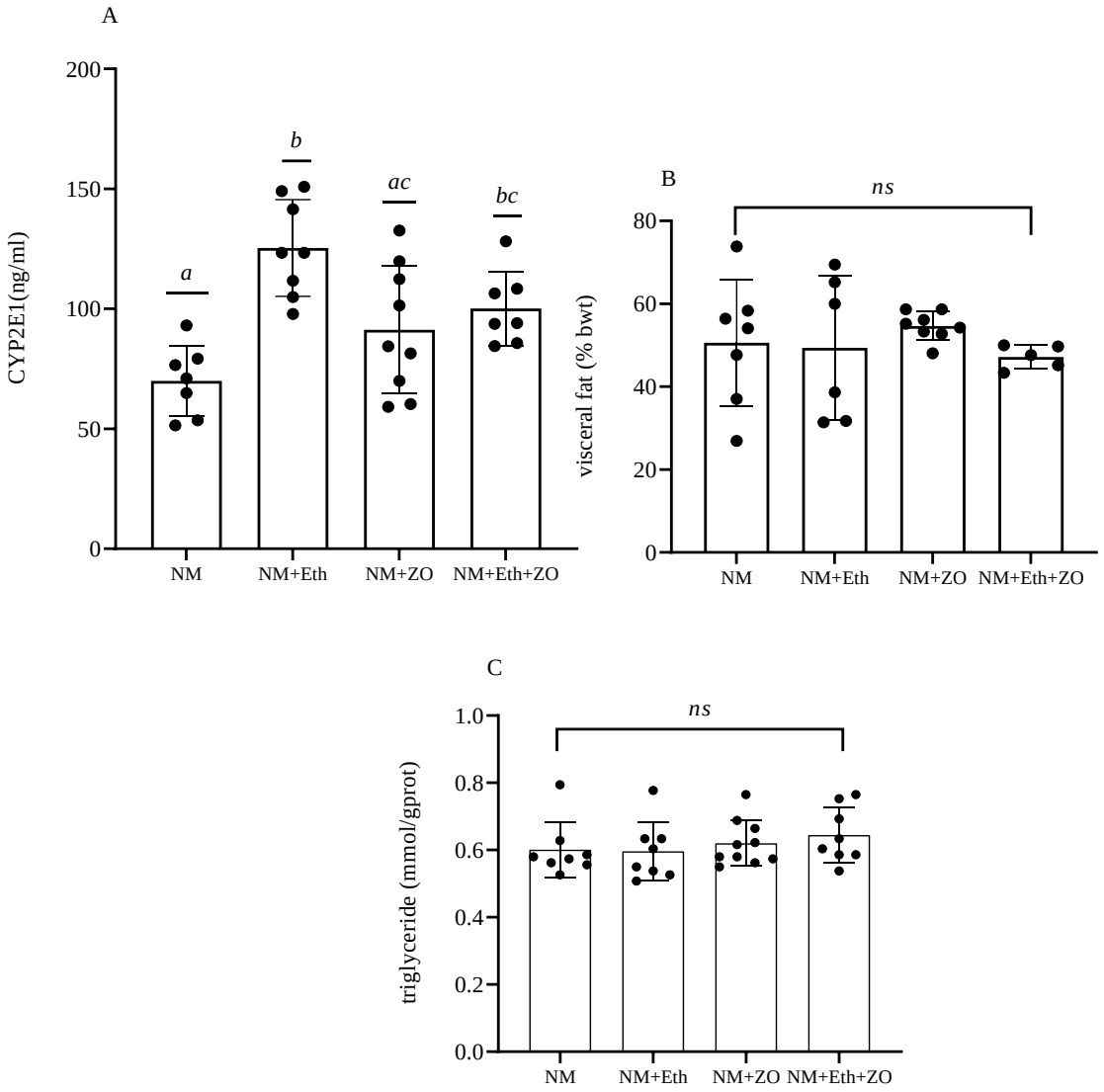

Figure 3.4: Effect of neonatal oral administration of zingerone on (A) liver cytochrome p450, (B) visceral fat mass (% b.wt) and (C) and triglycerides of male rat pups exposed to alcohol

Data presented as mean ± SD. ns = $p > 0.050$ when a statistical comparison is non-significant across treatment groups, ^{ab} = bar means with different letters significantly different at $p < 0.050$. NM=gavaged with 10 mL/kg of nutritive milk: NM+Eth = gavaged with 1g/kg of alcohol in nutritive milk; NM+ZO = gavaged with 40 mg/kg bwt of zingerone in nutritive milk;

NM+Eth+ZO = gavaged with 1 g/kg bwt of alcohol + 40 mg/kg of zingerone in nutritive milk. n = 5-8 per treatment

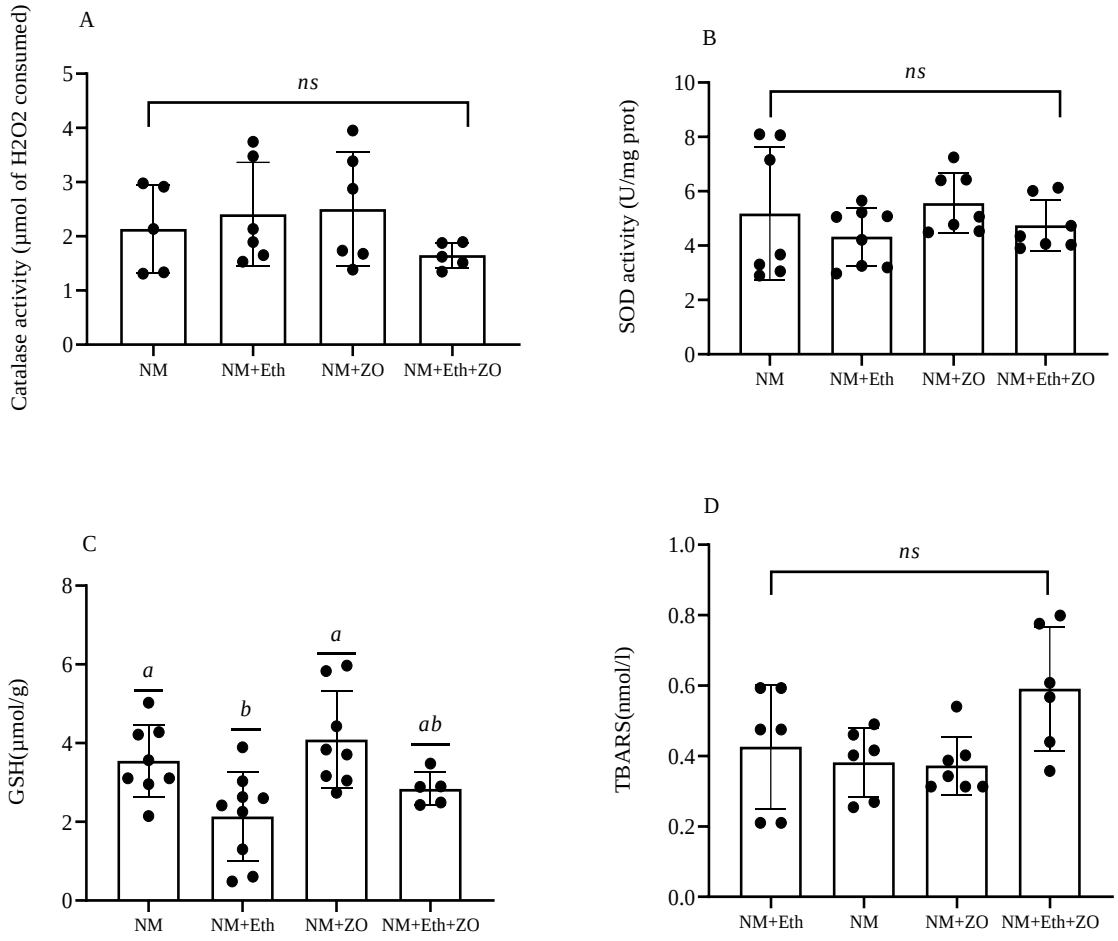

Figure 3.5: Effect of neonatal oral administration of zingerone on anti-oxidants (A) catalase activity (B) superoxide dismutase (C) glutathione proteins and liver oxidative stress (D) TBARS in male rat pups exposed to alcohol.

ns = $p > 0.050$ when the statistical comparison is non-significant across treatment groups. ^{ab} = bar means with different letters significantly different at $p < 0.050$. NM=gavaged with 10 mL/kg of nutritive milk: NM+Eth = gavaged with 1 g/kg bwt of alcohol in nutritive milk; NM+ZO = gavaged with 40 mg/kg zingerone in nutritive milk; NM+Eth+ZO = gavaged with 1 g/kg bwt of alcohol + 40 mg/kg bwt of zingerone in nutritive milk. n = 5-8 per treatment

3.4.4 Effects on plasma lipids, glucose, leptin and insulin concentration, hepatic triglycerides and HOMA-IR

Treatment regimens had no effect on the lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol) of both female and male rat pups ($p > 0.05$; Table 3.3), except for plasma TG. In female rat pups, when compared to control (NM), the oral administration of alcohol (NM+Eth) significantly reduced ($p = 0.044$; Table 3.3) TG concentration. TG concentration was not significantly different in the male rat pups ($p = 0.202$; Table 3.3). Neonatal oral administration with ZO ($p = 0.305$; Table 3.3) and combined Eth and ZO ($p = 0.995$; Table 3) did not affect plasma TG concentration.

Visceral fat mass relative to per cent body mass was not statistically different across treatment regimens for females ($p = 0.523$; Fig 3.2B) and males ($p = 0.704$; Fig 3.4B). Liver TG concentration was similar in female and male rat pups ($p = 0.213$; Fig 3.2C, $p = 0.612$; Figure 3.4C) across treatment regimens. Table 3.4 shows the effect of the treatment regimens on the rat pups' blood glucose, plasma leptin and insulin concentration and HOMA-IR. Neonatal oral administration of alcohol (NM+Eth), zingerone (NM+ZO) and combined alcohol and zingerone (NM+Eth+ZO) had no effect on the rat pups' blood glucose, plasma insulin and leptin concentration and HOMA-IR ($p > 0.05$; Table 3.4).

Table 3.3: The effect of neonatal oral administration of zingerone on lipid profile in suckling rat pups exposed to alcohol

Parameter	Sex	NM	NM+Eth	NM+ZO	NM+Eth+ZO
TG (mmol/L)	Female	0.85±0.21[a]	0.56±0.15[b]	0.69±0.19[ab]	0.84±0.23[ab]
	Male	0.88±0.37[a]	0.71±0.13[a]	0.68±0.19[a]	0.90±0.34[a]
HDL (mmol/L)	Female	1.63±0.46[a]	1.78±0.44[a]	1.62±0.41[a]	1.85±0.38[a]
	Male	1.68±0.42[a]	1.61±0.42[a]	1.66±0.60[a]	1.71±0.51[a]
TC (mmol/L)	Female	2.49±0.76[a]	2.35±0.60[a]	2.37±0.65[a]	2.62±0.91[a]
	Male	2.66±0.91[a]	2.34±0.50[a]	2.25±0.39[a]	2.87±0.83[a]
LDL (mmol/L)	Female	1.19±0.96[a]	1.82±0.71[a]	1.23±0.45[a]	1.38±0.29[a]
	Male	1.35±0.54[a]	1.19±0.37[a]	0.74±0.32[a]	1.43±0.79[a]

Data presented as mean ± standard deviation. p-value was set at $p < 0.050$. ab = within row means with different letters significantly different at $p < 0.050$. NM=gavaged with 10 mL/kg bwt of nutritive milk: NM+Eth = gavaged with 1g/kg bwt of alcohol in nutritive milk; NM+ZO = gavaged with zingerone 40 mg/kg bwt in nutritive milk; NM+Eth+ZO=gavaged with 1 g/kg bwt of alcohol + 40 mg/kg bwt of zingerone in nutritive milk. TG=Triglycerides; HDL-high density lipoprotein cholesterol; TC= Total cholesterol; LDL=Low density lipoprotein cholesterol.

Table 3.4: The effect of neonatal oral administration of zingerone on glucose and leptin in suckling rat pups exposed to alcohol

Parameter	Sex	NM	NM+Eth	NM+ZO	NM+Eth+ZO
Glucose (mmol/L)	Female	5.74±0.53	5.49±0.39	5.25±0.82	5.56±0.80
	Male	5.41±0.37	5.48±1.27	5.90±0.53	5.96±0.99
Insulin (ng/mL)	Female	1.10±0.47	1.29±0.24	1.08±0.36	1.26±0.52
	Male	1.10±0.32	0.99±0.32	1.31±0.17	0.95±0.23
HOMA-IR	Female	0.37±0.18	0.30±0.10	0.30±0.09	0.40±0.18
	Male	0.35±0.18	0.31±0.15	0.44±0.12	0.30±0.13
Leptin (ng/mL)	Female	0.91±0.21	0.88±0.16	0.80±0.12	0.90±0.23
	Male	0.89±0.27	0.90±0.13	0.75±0.25	1.05±0.28

Data presented as mean ± standard deviation $p > 0.050$. NM = gavaged with 10 mL/kg bwt of nutritive milk: NM+Eth = gavaged with 1g/kg bwt of alcohol in nutritive milk; NM+ZO = gavaged with zingerone 40 mg/kg bwt in nutritive milk; NM+Eth+ZO = gavaged with 1 g/kg bwt of alcohol + 40 mg/kg bwt of zingerone in nutritive milk. HOMA-IR = homeostatic model assessment of insulin resistance. n = 8-10 per treatment

3.5 Discussion

In this study, we investigated the potential of neonatal orally administered zingerone to protect against the negative impact of neonatal alcohol-induced oxidative stress in male and female Sprague Dawley rats. We showed that neonatal alcohol exposure (NAE) in rat pups induces oxidative stress, which may have a long-lasting impact on metabolic profile in adulthood. A sexually dimorphic outcome was observed in response to NAE: alcohol induced elevated CYP2E1 concentration and reduced total glutathione in male rat pups, while in female rat pups

it elicited a reduction in plasma TG. Zingerone did not affect growth performance, hepatic CYPE21 concentration and triglycerides, plasma lipids and blood glucose metabolism. Still, zingerone failed to protect against the alcohol-induced hepatic increase in CYP2E1 and decrease in glutathione concentration.

In this study, body mass was unaffected by any treatment regimen. Body mass can be affected by hydration and feeding status; hence, other growth performance indicators are preferred (Sundström et al., 2014). However, we did not find any significant effect of NAE on the empty carcass and long bone indices, indicating that NAE did not affect growth performance. In a comparable study in which rat pups received alcohol through breastfeeding, no change in body mass was recorded (Chen & Nyomba, 2004). Contrary, prenatal alcohol exposure decreased body mass because of nutrient deprivation (de Freitas et al., 2014; Cheng et al., 2021). However, using rat pups that can suckle from their dams along with the intervention in this study did not affect caloric intake. Additionally, the ethanol was not administered to the dams. Ethanol administration to dams affects maternal care and decrease milk production (Pepino et al., 2007; Ponce et al., 2011). Furthermore, we observed that the rat pups opened their eyes by PND 15 and nibbled on the rat chow available to the dams; thus, in addition to maternal milk and nutritive milk, the pups also got some of the energy from the rat chow.

Zingerone did not affect body mass and empty carcass. Other studies have reported that ZO does not affect body mass in adult rats (Cui et al., 2018; Muhammad et al., 2021). Decreased bone mass is related to decreased bone formation due to reduced osteocalcin, IGF-1 and vitamin D (González-Reimers et al., 2005). However, ZO did not affect long bone indices, suggesting that ZO may have osteogenic properties as previously reported by Srinaath et al. (2019).

Nutrition and hepatic pathology affect liver mass; an increase in liver mass can result from a fatty liver, while a decrease can be due to undernutrition (Pandit & Gupta, 2019). Since the liver is the center of ethanol metabolism, we examined its gross morphometry and possible oxidative damage. The absolute liver mass and its mass relative to the tibiae length regardless of treatment or sex was unaffected. This agreed with our earlier speculation that NAE and zingerone did not affect the nutritional intake of the rat pups. Malnutrition results in hypoleptinemia (Paillaud et al., 2022). However, leptin was also not affected by any of the treatment interventions.

Alcohol-treated male rat pups had an elevated CYPE21 concentration. Generally, CYP2E1 is induced during excess alcohol consumption, but other studies suggest that the microsomal ethanol-oxidizing system (MEOS) induced by CYP2E1 and catalase may be the primary ethanol metabolizing enzyme even at low ethanol concentration (Takagi et al., 1986; Peana et al., 2017). In support of our findings, Chhabra et al. (1996) demonstrated that rat pups exposed to alcohol through their dams' milk induced liver CYP2E1; however the induction was significantly high in male rat pups compared to female counterparts. The induction of CYP2E1 is associated with the generation of reactive oxygen species, which can induce oxidative stress (Bhatia et al., 2019).

In the current study, zingerone did not induce CYP2E1. The potent anti-oxidant and anti-inflammatory properties of ZO might have modulated the production of CYP2E1 (Bashir et al., 2021). Previous report demonstrated that pre-treatment with ZO blunts elevation of CYP2E1 transcripts when exposed to Cisplatin (Cis) or γ-Irradiation (IR)-induced hepatotoxicity in adult male albino rats (Mohamed & Badawy, 2019). Hepatic catalase activity was similar in the rat pups across treatment regimens. This result was unexpected, especially in the alcohol-treated group, as catalase plays an accessory role in ethanol metabolism. However contradictory results concerning catalase activity in rat pups have been reported by

Ojeda et al. (2009) who observed an increased activity, while de Freitas et al. (2014) reported a decrease in catalase activity. Our results suggest that catalase may not be actively involved in hepatic ethanol metabolism, as a previous study indicates that the pharmacokinetics of ethanol in liver cells does not involve catalase (Aragon et al., 1989).

In the current study, SOD activity was not affected by the neonatal treatments. This finding contradicts other studies (Cheng & Kong, 2011; Kołota et al., 2020). However, this variance might relate to the age of the animals, as they used adult rats, unlike the neonate rats used in this study. Ageing is associated with a decline in mitochondrion enzyme activities involved in scavenging free radicals (Kaplán et al., 2019). Related studies with rat pups that received ethanol during lactation also reported that NAE did not affect activity (Ojeda et al., 2009; de Freitas et al., 2014).

Our findings point to alcohol-induced reduction in total glutathione (GSH) concentration in the male rat pups. Previous studies observed a decrease in hepatic glutathione peroxidase (GPx) and glutathione-S-transferase (Ojeda et al., 2009; de Freitas et al., 2014). Glutathione peroxidase activity correlates positively with GSH levels, as GSH serves as an essential co-factor for GPx activity. Alcohol inhibits the transport of cytosolic GSH into the mitochondrion leading to depletion that affect the amount of glutathione needed to bind acetaldehyde (Fernández-Checa, 2003). This allows for the formation of excessive amounts of free radical species, causing oxidative stress. We observed that alcohol induces a reduction in glutathione concentration in a sexually dimorphic manner as only males had a reduced concentration. This effect may be related to estrogens in females (Lavoie & Tremblay, 2018); estrogens have been shown to induce glutathione metabolism and contribute significantly to sex specificity in oxidative stress because of it (glutathione) controls intracellular levels of peroxides, aldehydes and radicals (Lavoie & Tremblay, 2018). It is noteworthy that both males and females are

exposed to high levels of estrogen *in-utero*; however, there is a perinatal surge in testosterone in the first week of the rodent life (Dearden et al., 2018), thus reducing estrogen functionality.

Despite the above observation, there was no significant increase in the hepatic TBARS concentration in the alcohol-exposed pups. A previous study reported similar findings (de Freitas et al., 2014). In lipid peroxidation, free radicals or non-radical species attack lipids containing carbon-carbon double bond(s), especially the C-C double bonds of polyunsaturated fatty acids (Ayala et al., 2014). Perhaps the small amount of fat content of the rat pups contributed to the non-detection of lipid peroxidation (Tavares do Carmo et al., 1999). However, we did not determine cholesterol oxidized products.

Orally administered zingerone only maintained anti-oxidant ability and prevented an elevation in CYP2E1 concentration comparable to the control. Zingerone is a polyphenol, and by its (ZO) redox properties can act as free radical quenchers, reducing agents, hydrogen donors, metal chelators, and decomposers of peroxides hence preventing oxidative stress (Bashir et al., 2021). However, zingerone combined with alcohol did not influence CYP2E1 and glutathione concentration. Aeschbach et al. (1994) demonstrated that although zingerone has anti-oxidant properties, its inhibitory action on oxidative stress is weak relative to other components of ginger like geraniol, and 6-gingerol. Meanwhile, studies have also reported that zingerone does not affect GSH and lipid peroxidation (Aeschbach et al., 1994; Eid et al., 2017). Additionally, besides the short duration of administration, the co-administration of alcohol with zingerone might have reduced the anti-oxidative potential of zingerone. Previous pre-clinical studies investigating the association between early-life and offspring metabolic health typically use a high dose [2 g kg^{-1} b.w.] administered twice a day (Akison et al., 2019) throughout gestation and during lactation. This likely increased blood alcohol availability and thus the pronounced metabolic effect observed in those studies. The low, acute dose model that we used in the

current study mimics the amount of alcohol that would be consumed by the breastfeeding infants of lactating women who report drinking on average one or two standard drinks per day.

In this study, NAE had no effect on plasma insulin and insulin sensitivity of the rat pups. The low dose short-term alcohol administration used in this study possibly reduced insulin concentration and improved insulin sensitivity, particularly in females compared to males, as previously reported by other studies (Schrieks et al., 2015)**.** Zingerone did not affect insulin concentration in the rat pups; however, in diabetic rats and humans, treatment with zingerone reduces insulin levels and maintains normal glucose concentrations (Shidfar et al., 2015; Cui et al., 2018). The anti-diabetic effect of zingerone is likely due to its ability to activate peroxisome proliferator-activated receptors (*PPARs)* expression (Chung et al., 2009), which play an essential role in glucose and lipid homeostasis (Hassan et al., 2021).

Alcohol-treated female rat pups had significantly reduced plasma triglycerides, similar to the study of Gårdebjer et al. (2018), but tissue TG was unaffected. It is possible that the fat was being redistributed to peripheral tissue resulting in hypotriglycedemia as has been reported in adult rats that consumed alcohol (Steiner & Lang, 2017). Plasma and tissue lipids were unaffected by zingerone treatment. Studies show that suckling rats have scanty adipose tissue thus the amount of free fatty acids and glycerol released into circulation is not quantitatively relevant (Tavares do Carmo et al., 1999). Hence the effect of the interventions on plasma lipids cannot be considered relevant in rat pups.

In vitro studies show that ZO scavenges free radicals in a dose-dependent manner; the higher the dosage, the better its scavenging potential (Rao et al., 2009). Mani et al. (2016) administered 40 mg/kg bwt zingerone to adult Wistar rats which was effective at potentiating the anti-oxidant system of the rats against alcohol-induced oxidative stress. This dosage of zingerone (40 mg/kg bwt) was previously used in rat pups and this dosage was found

to be effective at preventing metabolic dysfunction in adulthood (Muhammed, 2021). Zingerone has a pungent taste that can activate transient receptor potential cation channel subfamily V member 1 (TRPV1), the receptor for heat and pain (Komai et al., 2006). Thus, investigators were concerned that a higher zingerone dose might cause the rat pups gastric discomfort and consequently impact acceptability by the rat pups. As such we used 40 mg/kg bwt as previously reported by other researchers conducting similar studies. Although a higher zingerone dosage might have been effective.

3.6 Conclusion

Due to reduced adiposity in suckling rat pups, the pups had stronger resistance to developing metabolic disorders associated with metabolic syndrome (Ghezzi et al., 2012). However, low-dose NAE induced hepatic oxidative stress via elevating CYP2E1 and decreasing GSH concentrations in males only, while orally administered zingerone did not affect hepatic CYPE21, anti-oxidant status and growth performance. Therefore, ZO may be administered as a safe alternative drug for oxidative-stress related conditions in neonates and this may confer positive health status in adulthood.

In this neonatal growth phase, while intervening with orally adiminstered zingerone did not protect the rat pups against alcohol-induced hepative oxidative stress, its (neonatal orally administered zingerone) effects in adult rats subjected to second alcohol insult needed to be explored. Thus the next experimental chapter explored the potential prophylactic effects of zingerone against alcohol-induced meatabolic derangements in rats modelling humans subjected to a double alcohol hit; through maternal breast milk and in adulthood through partaking in alcoholic beverages

3.7 References

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CHAPTER FOUR – THE EFFECT OF NEONATAL ZINGERONE ON BLOOD METABOLIC PARAMETERS IN CHRONIC ALCOHOL-TREATED RATS IN EARLY ADULTHOOD

4.0 Abstract

Objective: Early-life exposure to alcohol predisposes to metabolic disorders. Additional insult with alcohol in adulthood could exacerbate the effect of the early-life exposure. We, therefore, examined the potential of neonatal oral administration of zingerone, a phytochemical with anti-oxidant properties, for long-term protection against the development of alcohol-associated metabolic derangements.

Method: One hundred and twenty-three 10-day-old male (n=60) and female (n=63) Sprague-Dawley rat pups were randomly assigned and administered the following treatment regimens daily from postnatal day (PND) 12-21: group 1 - nutritive milk (NM), group 2 – NM +1g/kg ethanol (Eth), group 3 - NM + 40 mg/kg ZO, group 4 – NM + Eth +ZO. After weaning, on PDN 22, each group was divided into 2 subgroups: subgroup I had tap water and subgroup II had alcohol solution as drinking fluid for eight weeks. Weekly body mass, feed, fluid and calorie intake were measured. Fasting plasma total cholesterol, triglycerides, high-density cholesterol, adiponectin, insulin, leptin and blood glucose and concentration and visceral fat mass were quantified.

Results: Male and female rats that had alcohol as the drinking fluid in adulthood had a decreased feed and fluid intake, but caloric intake was increased ($p < 0.050$) compared to control (NM+W^{ad}). In both sexes, an early single, late, and double hit with ethanol alone or together with neonatal orally administered zingerone neither affected body mass nor caused metabolic effects in adulthood ($p > 0.05$). Blood glucose was decreased in late single hit male rats ($p = 0.026$ vs control). However, neonatal orally administered zingerone had no effect (p

> 0.05) on plasma glucose concentration in male adult rats given alcohol as a drinking fluid. In females, neonatal orally administered ZO and late single hit alcohol significantly reduced visceral fat mass ($p = 0.045$ vs control (NM+W^{ad}).

Conclusion: We conclude that neonatal orally administered zingerone mitigated ethanol-induced decrease in blood glucose concentration in male rats and visceral fat mass accumulation in female rats. Thus, zingerone can potentially be administered during the neonatal period to protect against alcohol-associated metabolic dysfunctions.

Keywords: Zingerone, alcohol, neonatal, adulthood, visceral fat mass, glucose

4.1 Introduction

The metabolic system is necessary for survival and plays a central homeostatic role in humans. Its disruption can lead to varied chronic metabolic disorders including type 2 diabetes, cardiovascular disease, obesity and fatty liver disease (Hotamisligil, 2006; Smith et al., 2018). The aetiology of metabolic disorders remains elusive; however, external factors such as alcohol consumption, smoking, diet and physical activity are modifiable factors that can potentially prevent the condition (Han et al., 2020).

Adult susceptibility or resistance to metabolic disorders is traceable to early life events referred to as the developmental origins of health and disease (Fall & Kumaran, 2019). According to the developmental origins of health and disease (DOHaD) hypothesis, the early growth phases, including the lactation period, are critical periods of metabolic plasticity where sub-optimal nutritional stimuli can cause epigenetic changes that can result in either an increased or decreased propensity to develop metabolic disorders later in life (Ellsworth et al., 2018). Some 80% of breastfeeding mothers return to drinking alcohol following a period of abstinence (Tran et al., 2015) during pregnancy due to awareness of alcohol-induced risks to the foetus (Popova

et al., 2022). Exposure to single or multiple insults during the critical periods of plasticity could programme a person for metabolic disorders (Agosti et al., 2017). Prenatal and lactation alcohol exposure cause oxidative stress, intrauterine growth restriction (IUGR) with concomitant activation of resistin and lipid transcript genes sterol regulating element-binding protein and fatty acid synthase (Chen & Nyomba, 2003a, 2004; Freitas et al., 2014; Shen et al., 2014). These lipid regulating genes may remain activated into adulthood leading to the development of dyslipidaemia and obesity (Magee et al., 2008). Additionally, early-life exposure to alcohol increases the propensity to drink alcohol in adolescence due to appetitive learning induced by the sensory and reinforcing pharmacological properties of alcohol (Gaztañaga et al., 2020). Hence exposure to alcohol at early-life can be the first insult and adulthood alcohol consumption a second insult (Gaztañaga et al., 2020). Yet to the best of our knowledge most studies that investigated the impact of prenatal alcohol exposure in adult rats used a high-fat diet as the second insult (Akison et al., 2019). A controversial relationship exists between alcohol consumption and metabolic disorders as it depends on the quantity, duration and pattern of alcohol consumption (Lee et al., 2017; Yun et al., 2021). Interestingly alcohol has a dual effect on lipid metabolism: it stimulates lipolysis of adipose tissue lipolysis and but promotes hepatic *de novo* lipogenesis (Shen et al., 2019). Alterations in lipolysis and lipogenesis are associated with insulin resistance (Saponaro et al., 2015), a central factor in metabolic disorders. Additionally, alcohol consumption increases the expression of inflammatory cytokines and chemokines, creating an inflammatory milieu as occurs in high-fat diet-induced obese rodents (Souza-Smith et al., 2016) but without a necessary increase in adipocyte size (Gregor & Hotamisligil, 2011).

The management of metabolic disorders depend on curative approaches such as treatment with conventional pharmacological agents supported by adopting healthy lifestyle choices. The

concept of DOHaD opens an opportunity to strategically intervene during the critical periods of developmental plasticity to prevent the development of adverse developmental programming outcomes. Several studies show that neonatal administration of pharmacological and ethnomedicinal compounds can protect against disease development in adulthood (Madrigal-Santillán et al., 2014; Nyakudya et al., 2018; Muhammad et al., 2021). Zingerone (ZO) is a non-toxic phenolic compound derived from dried ginger (Ahmad et al., 2015). It has varied pharmacological activities, including anti-diabetic, anti-obesogenic, anti-hyperlipidaemic, and anti-inflammatory (Ahmad et al., 2015; Mani et al., 2016, Mani et al., 2017). Therefore, we interrogated the potential of neonatally administered ZO to protect against metabolic derangements associated with early and late single hit and double hit alcohol consumption in male and female rats.

4.2 Materials and methods

4.2.1 Study setting and ethics clearance

The study was undertaken at the Wits Research Animal Facility of the University of Witwatersrand. The Wits Animal Research Ethics Committee approved all protocols and experimental procedures (Ethical clearance number: 2019/10/57/B). We complied with accepted laboratory animal use and care principles stipulated in the South African National Standard (SANS 10386:2008) and Animals Protection Act, 1962: Act No. 71.

4.2.2 Animal housing and management

This study used 123 (male = 60; female = 63), 10-day-old Sprague-Dawley (SD) rat pups (*Rattus norvegicus*) from 21 nulliparous dams. The rat pups were culled to a minimum of 8 and a maximum of 12 per litter to minimise litter size effect (Suvorov & Vandenberg, 2016). Additionally, we ensured that only one rat pup per sex was assigned to an experimental group

according to the recommended experimental design of DOHaD studies (Dickinson et al., 2016). Pre-weaning, the rat pups were housed in acrylic cages with their dams and allowed to nurse freely from their respective dams until weaning. Post-weaning, the rats were housed individually in acrylic cages. Throughout the study, wood shavings were used as bedding and were changed twice a week. Room temperature in the animal house was kept at 24±2°C and a 12:12-hour light-dark cycle and adequate ventilation were maintained throughout the study. Standard rat chow (Epol®, Johannesburg, South Africa) and drinking fluids were made available *ad libitum* to the rats throughout the experiment.

This study used rats as they are altricial species hence provide a critical window of opportunity, during suckling, for neonatal reprogramming (Rabadán-Diehl & Nathanielsz, 2013). In rats, postnatal days (PNDs) 10-20 correspond to neonatal (suckling) period in humans (Picut et al., 2015) and a period of rapid differentiation and development of the liver. Nutritive milk [Kitty milk (V 16752 Act 36/1947); Kyron Labs, Johannesburg, South Africa] was used as the vehicle for alcohol (ACE; Johannesburg, South Africa) and zingerone [Sigma-Aldrich (W312401); Johannesburg, South Africa]. Oral gavage was administered at a dose of 10 mL/kg for all groups.

4.2.3 Sample size calculation

The one-way ANOVA formula for sample size determination as described by Arifin & Zahiruddin (2017) was used to estimate the sample size.

4.2.4 Study design

Figure 4.1 is a diagrammatic representation of the experimental design. The experiment was divided into three stages: neonatal intervention, no intervention, and adolescent intervention. The neonatal experimental design has previously been described. Briefly, the neonatal stage lasted from PND 12 – 21, a stage equivalent to the suckling stage in humans (Picut et al., 2015). One hundred and twenty-three, 10-day old SD rat pups were habituated to handling for two

days and then randomly allocated to and orally gavaged daily with the following treatments from PND 12-21:

- control - orally gavaged 10 mL/kg body mass of nutritive milk (NM).
- ethanol - orally gavaged 1 g/kg of ethanol (NM+ Eth).
- zingerone - orally gavaged 40 mg/kg/bwt zingerone (NM+ZO).
- ethanol + zingerone - orally gavaged with 40 mg ZO + 1 g/kg of ethanol (NM+Eth+ZO).

The second stage (no intervention) lasted from PND 22 – 45, where the weaned rat pups had *ad libitum* access to standard rat chow and plain drinking water and were allowed to reach adolescence (Spear and Swartzwelder, 2014).

Humans experiment with alcohol during adolescence (Marshall, 2014; Spear, 2014) hence the third experimental stage started from PND 46-100, a growth phase in rats equivalent to late adolescence to adulthood in humans (Picut et al., 2015). At the beginning of the third experimental (adolescence) stage, all rats from each stage I (neonatal) group were subdivided into two subgroups wherein subgroup I had unlimited access to plain drinking water and a standard rat chow (SRC) and subgroup II ethanol solution and an SRC. Feed and drinking fluid were availed *ad libitum*. Thus, there were eight groups at the third stage (Fig 4.1). The rats

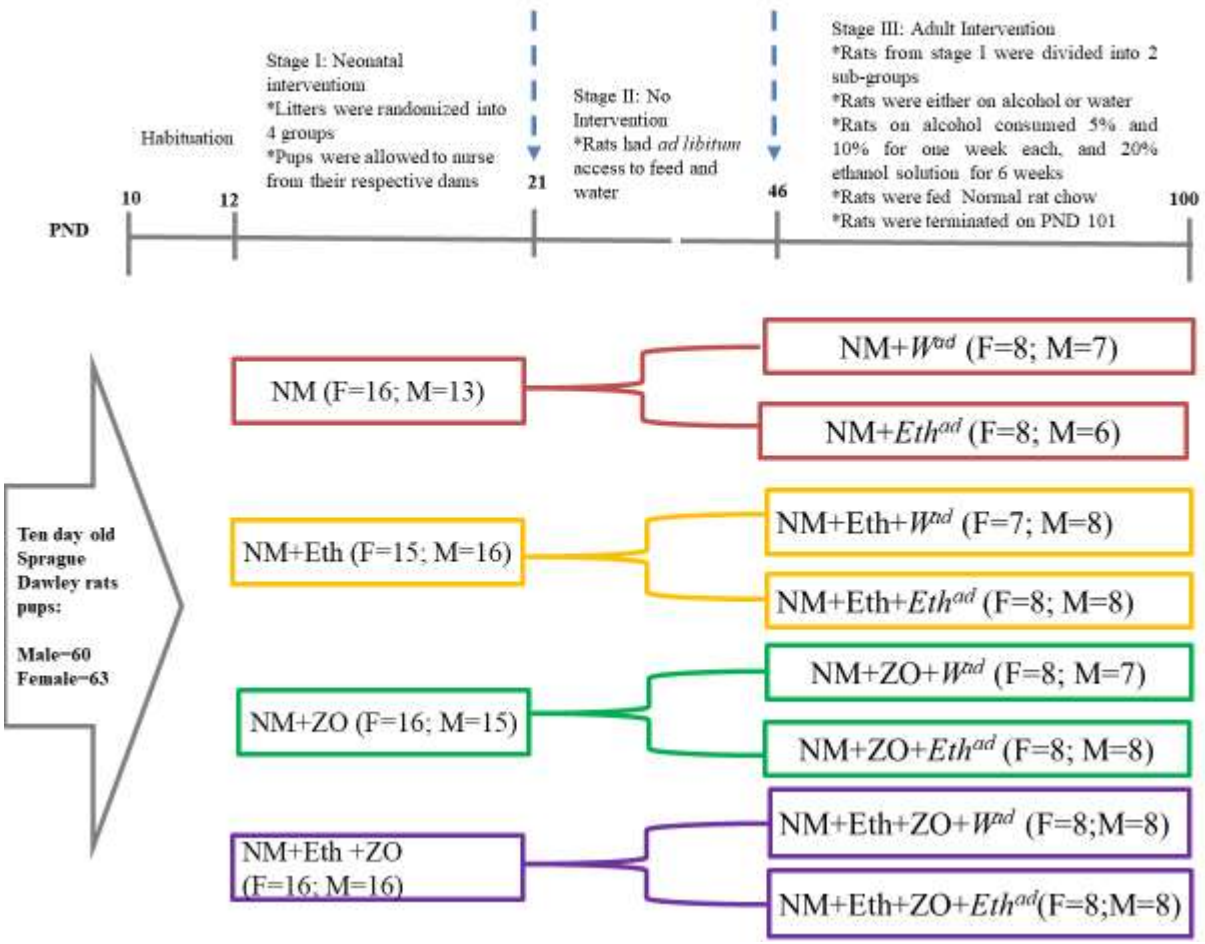

Figure 4.1: Experimental design of the study

control (NM+W^{ad}) – neonatally gavaged with nutritive milk (NM) and received plain drinking water (W^{ad}) at stage III; **early single hit (NM+Eth+W^{ad})** - neonatally gavaged with ethanol in nutritive milk and received plain drinking water (W^{ad}) at stage III; **late single hit (NM+Eth^{ad})** - neonatally gavaged with NM and received ethanol (Eth^{ad}) solution at stage III; **double hit (NM+Eth+Eth^{ad})** - neonatally gavaged with Eth in nutritive milk and received Eth^{ad} at stage III; **zingerone alone (NM+ZO+W^{ad})** - neonatally gavaged with ZO in nutritive and received plain drinking water (W^{ad}) at stage III; **early single hit with zingerone (NM+Eth+ZO+W^{ad})** - neonatally gavaged with NM, Eth, ZO and received plain drinking water (W^{ad}) at stage III; **late single hit with zingerone (NM+ZO+Eth^{ad})** - neonatally gavaged with NM and ZO and received Eth^{ad}

at stage III; **double hit with zingerone (NM+Eth+ZO+*Eth*^{ad})** - neonatally gavaged with NM, Eth and ZO and received *Eth*^{ad} at stage III; F-Female; M-Male; NM-Nutritive milk; Eth-ethanol; ZO-zingerone; W-water

from each subgroup II received a 5% (v/v) ethanol solution from PND 46-52, 10% (v/v) ethanol solution from PND 53-59 and 20% (v/v) ethanol solution from PND 60-100. This alcohol protocol has been shown to cause alcoholic fatty liver disease and can subsequently impact rats' metabolic parameters (Ojeda et al., 2008). On PND 100, the rats were fasted overnight and euthanised on PND 101.

4.2.5 Fluid, feed and calorie intake

Feed and fluid intake were determined weekly during stage III of the experiment. Each rat's feed and fluid refusals were measured and subtracted from the respective total offered. Fluid intake represents total amount of either water or ethanol solution dependent on the group. Weekly feed and fluid intake was expressed as a percentage of body mass; g/100g and mL/100g, respectively. The nutrient composition of the commercial SRC used is provided as supplementary data S1. Total weekly calorie intake was determined as follows:

$$Weekly\ total\ calories = weekly\ ethanol\ grams\ in\ solution\ consumed\ \left(\frac{mL}{100g}\right) \times ethanol\ calorie\ value + weekly\ feed\ intake\ \left(\frac{g}{100g}\right) \times feed\ calorie\ value$$

Ethanol calorie value = 7.0929 Kcal (Veale & Myers, 1968); Feed calorie value = 2.8Kcal (Muhammed et al., 2021).

4.2.6 Body mass measurement

Body mass was determined using a weighing scale (Snowrex Electronic Scale, Clover Scales, Johannesburg, South Africa) to monitor growth performance and adjust the dosage of the treatments. The rat pups were weighed daily at pre-weaning and weekly post-weaning.

4.2.7 Terminal procedures and tissue collection

Following an overnight fast, on PND 101, each rat was weighed on an electronic balance (Presica 310M, Presica Instruments AG, Switzerland). A blood drop was obtained via tail vein pinprick to determine fasting blood glucose concentration using a calibrated blood glucose meter (ContourPlus Bayer Health Care, Isando, South Africa). The rats were euthanased via intraperitoneal injection (200 mg/kg body mass) with sodium pentobarbital (Eutha-naze®, Bayer, Johannesburg, South Africa) and then carefully cut open by a midline incision. Blood was then collected via cardiac puncture using 18 G needles (Becton Dickinson Vacutainer Systems Europe, Meylan Cedex, France) and 10 mL syringes into heparinised blood collection tubes. Plasma was obtained after centrifugation of the blood at 3 000 × g for 15 mins and stored at -80 °C in microtubes until use. Visceral fat surrounding the liver, kidney, pancreas, stomach, small and large intestines was carefully removed and weighed on a Presica 310M digital scale (Precision Instruments, Johannesburg, South Africa).

4.2.8 Determination of plasma lipids profile

Plasma triglyceride, total cholesterol and high-density lipoprotein cholesterol concentration were determined using colorimetric assay kits as per the manufacturer's instruction (Elabscience ®, Wuhan, Hubei Province, China). Low-density lipoproteins concentration was computed using the Friedewald equation (Knopfholz et al., 2014): $LDL - Cholesterol\ (\frac{mmol}{L}) = Total\ cholesterol - (HDL - cholesterol + \frac{Triglycerides}{2.2})$

4.2.9 Determination of plasma hormone concentration

Rat-specific insulin (INS), adiponectin (ADP) and leptin (LEP) ELISA kits (Elabscience®, Rat ELISA kit, Wuhan, Hubei Province, China) were used to determine the plasma insulin, adiponectin and leptin concentration, respectively, according to the manufacturer's instructions. Insulin resistance was computed based on the homeostasis model assessment of insulin resistance (Matthews et al., 1985):

$$HOMA - IR = \frac{[Fasting\ plasma\ insulin\ (\mu U\ /mL) \times Fasting\ glucose\ (mmol\ /L\ )]}{22.5}$$

4.3 Statistical analysis

GraphPad Prism 8 software (Graph-Pad Software Inc., San Diego, USA) was used to analyse the data. Data are 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. Statistical significance was considered when $P < 0.05$.

4.4 Results

4.4.1 Effect of neonatal orally administered zingerone on body mass

In male and female rats, there was a no statistically difference in body masses at induction [p > 0.184 (males); p = 0.178 (females)], weaning [p = 0.236 (males); p = 0.563 (females)], adolescence [p = 0.610 (males); p = 0.767 (females)], and terminal body mass [p = 0.056 (males); p = 0.093 (females)] across treatment groups (Figs 4.2 and 4.3). Early, late single hit

and double hit alcohol and neonatal ZO did not have an effect on the body masses of male and female rats ($p > 0.050$). Male and female rats grew significantly from induction to weaning to adolescence and adulthood regardless of neonatal treatment (Fig 4.2 and 4.3, $p < 0.0001$).

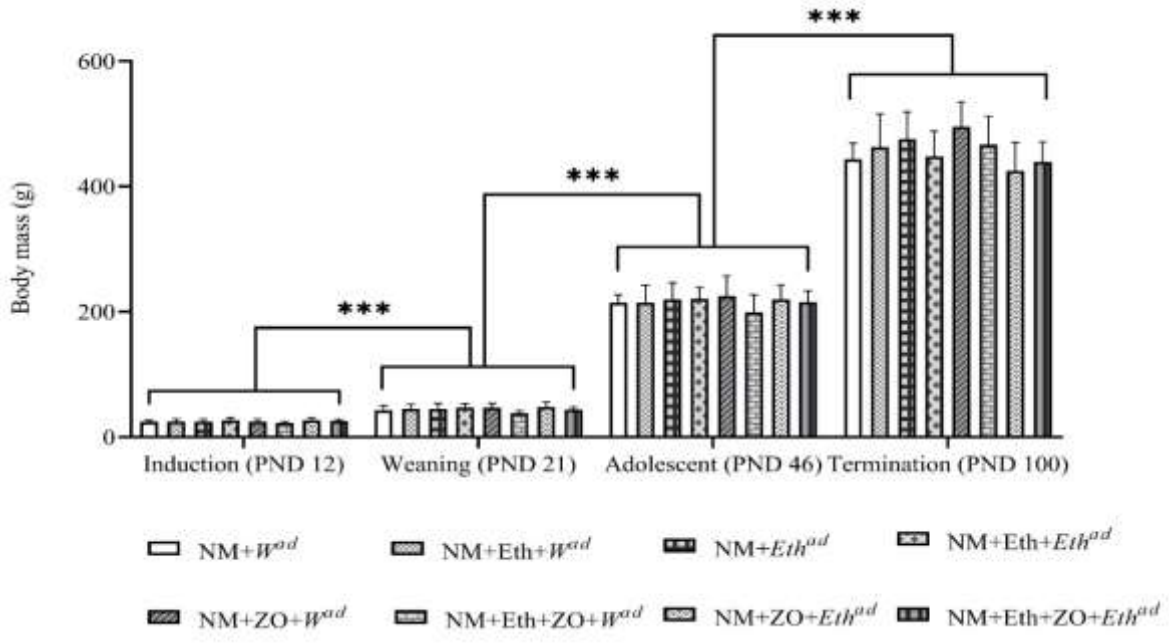

Figure 4.2: Body mass of male rats

Data is presented as mean ± standard deviation. ***Significant increase in body mass from induction to weaning, weaning to adolescence and from adolescence to termination at adulthood ($p < 0.05$). **control (NM+W^{ad})** – neonatally gavaged with nutritive milk (NM) and received plain drinking water (W^{ad}) at stage III; **early single hit (NM+Eth+W^{ad}) -** neonatally gavaged with ethanol in nutritive milk and received plain drinking water (W^{ad}) at stage III; **late single hit (NM+Eth^{ad}) -** neonatally gavaged with NM and received ethanol (Eth^{ad}) solution at stage III; **double hit (NM+Eth+Eth^{ad}) -** neonatally gavaged with Eth in nutritive milk and received Eth^{ad} at stage III; **zingerone alone (NM+ZO+W^{ad}) -** neonatally gavaged with ZO in nutritive and received plain drinking water (W^{ad}) at stage III; **early single hit with zingerone (NM+Eth+ZO+W^{ad}) -** neonatally gavaged with NM, Eth, ZO and received plain drinking water (W^{ad}) at stage III; **late single hit with zingerone (NM+ZO+Eth^{ad}) -** neonatally gavaged

with NM and ZO and received Eth^{ad} at stage III; **double hit with zingerone (NM+Eth+ZO+Eth^{ad})** - neonatally gavaged with NM, Eth and ZO and received Eth^{ad} at stage III; NM-Nutritive milk; Eth-ethanol; ZO-zingerone; W-water; n = 6-8 per treatment group

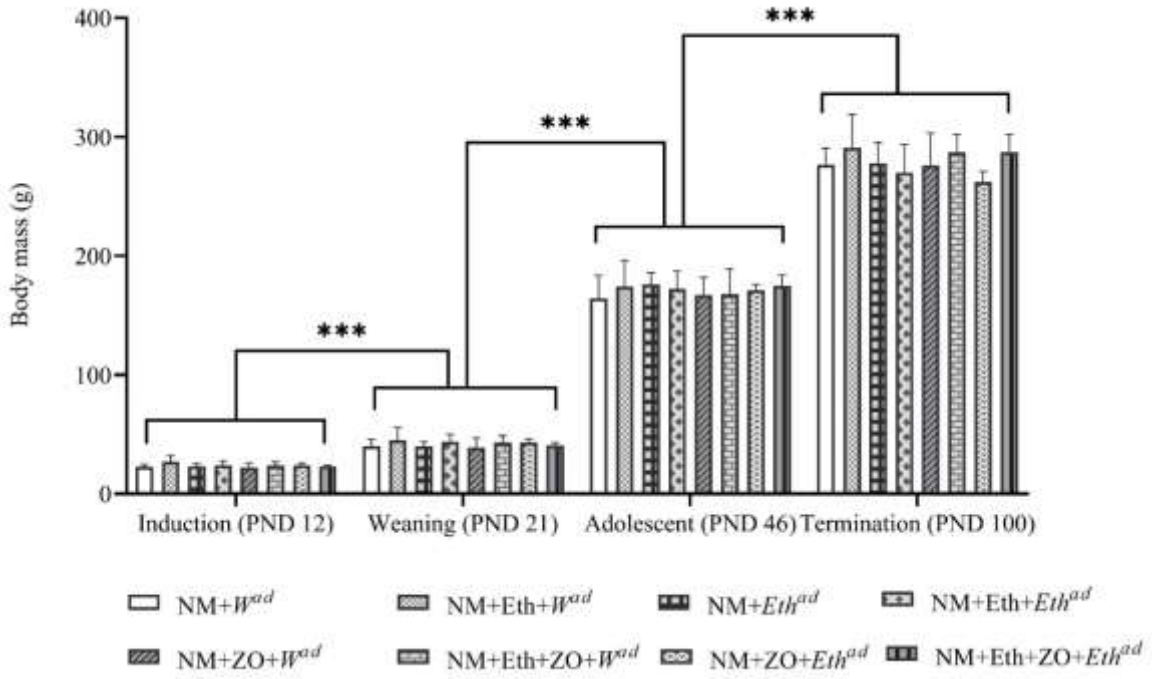

Figure 4.3: Body mass of female rats.

Data is presented as mean ± standard deviation. ***Significant increase in body mass from induction to weaning, weaning to adolescence and from adolescence to termination ($p < 0.05$). **control (NM+W^{ad})** – neonatally gavaged with nutritive milk (NM) and received plain drinking water (W^{ad}) at stage III; **early single hit (NM+Eth+W^{ad})** - neonatally gavaged with ethanol in nutritive milk and received plain drinking water (W^{ad}) at stage III; **late single hit (NM+Eth^{ad})** - neonatally gavaged with NM and received ethanol (Eth^{ad}) solution at stage III; **double hit (NM+Eth+Eth^{ad})** - neonatally gavaged with Eth in nutritive milk and received Eth^{ad} at stage III; **zingerone alone (NM+ZO+W^{ad})** - neonatally gavaged with ZO in nutritive and received plain drinking water (W^{ad}) at stage III; **early single hit with zingerone (NM+Eth+ZO+W^{ad})** - neonatally gavaged with NM, Eth, ZO and received plain drinking water (W^{ad}) at stage III; **late single hit with zingerone (NM+ZO+Eth^{ad})** - neonatally gavaged with NM and ZO and

received *Eth*^{ad} at stage III; **double hit with zingerone (NM+Eth+ZO+*Eth*^{ad}) -** neonatally gavaged with NM, Eth and ZO and received *Eth*^{ad} at stage III; NM-Nutritive milk; Eth-ethanol; ZO-zingerone; W-water; n = 6-8 per treatment group

4.4.2 Effect of neonatal orally administered zingerone on weekly feed, fluid, and calories intake

Table 4.1 shows the rats' overall weekly mean feed, fluid, and calorie intake during the third phase of the experiment. There was a statistical difference in the overall mean weekly feed ($p < 0.0001$ vs control; Table 4.1) and fluid ($p < 0.0001$ vs control; Table 4.1) intake across treatment groups for males and females. Alcohol consumption caused a significant reduction in the overall mean feed intake by the rats [$p < 0.0001$ vs control (males), $p < 0.0001$vs control (females)]. In male rats, feed intake started decreasing from week 2, but in females, feed intake decreased from week 1 ($p < 0.05$ vs control; Table S4.2A & S4.2B).

Alcohol consumption in male rats that were neonatally administered with alcohol (NM+Eth+*Eth*^{ad}, $p = 0.001$ vs control) and in combination with ZO (NM+Eth+ZO+*Eth*^{ad}, $p = 0.006$ vs control) caused a significant decrease in fluid intake. The male rats that were administered with a late single hit of alcohol had a similar fluid intake as the control ($p = 0.065$; Table 4.1A). However, neonatal orally administered ZO with a late alcohol single hit decreased fluid consumption ($p = 0.001$; Table 4.1). Significant fluid intake decrease was evident from week 3-8 for all rats that had ethanol solution as drinking fluid except the late alcohol single hit group ($p < 0.05$ vs control; Table S4.3A). In females, alcohol consumption significantly ($p < 0.05$ vs control; Table 4.1B) decreased fluid intake. Fluid intake started decreasing from week 2 of stage III through to week 8 ($p < 0.05$ vs control; Table S4.3B). Rats that received water in adulthood had a similar feed and fluid intake with the control for males and females ($p > 0.05$ vs control; Table 4.1).

In males, alcohol consumption increased calorie intake ($p < 0.05$ vs control; Table 4.1A) regardless of neonatal treatment. In rats that had alcohol in adulthood, significant calorie intake increases were evident from week 4 to 8 ($p < 0.05$ vs control; Table S4.4A). Similarly, alcohol consumption caused a significant calorie increase ($p < 0.050$ vs control; Table 4.1B) in female rats regardless of neonatal intervention. The double alcohol hit group showed a significant increase in calorie intake at weeks 4 and 8 only ($p = 0.010$, $p < 0.0001$ vs control; Table S4.4B). From week 6 to 8, the late alcohol single hit group showed a significant increase in calorie intake ($p = 0.034$, $p = 0.001$, $p < 0.0001$ vs control at week 6, 7 and 8, respectively; Table S4.4B). Neonatal ZO administration combined with a late alcohol single hit delayed increase in calorie intake ($p = 0.014$, $p < 0.0001$ vs control; Table S4.4B) until week 7.

Table 4.1: The effect of neonatal orally administered zingerone on fluid, feed and calorie intake in adult rats drinking alcohol in adulthood

A. Males

Parameter	Feed Intake (g/100g body mass)	Fluid Intake (mL/100g body mass)	Calorie Intake (Kcal/100g body mass)
NM+W^{ad}	39.97±1.62^{a}	79.95±8.18^{a}	111.9±4.53^{a}
NM+Eth+W^{ad}	39.15±2.56 ^{a}	82.74±6.34^{a}	109.6±5.57^{a}
NM+Eth^{ad}	30.78±1.73^{b}	65.10±7.62^{a}	130.0±9.38^{b}
NM+Eth+Eth^{ad}	29.63±1.12^{b}	55.91±9.72^{b}	127.9±9.27^{b}
NM+ZO+W^{ad}	40.66±1.20^{a}	79.95±8.18^{a}	113.9±3.36^{a}
NM+Eth+ZO+W^{ad}	40.62±2.57 ^{a}	79.29±6.93^{a}	111.8±4.72^{a}
NM+ZO+Eth^{ad}	30.05±1.54 ^{b}	59.55±11.13^{b}	132.2±10.91^{b}
NM+Eth+ZO+Eth^{ad}	29.67±1.68 ^{b}	59.72±8.29^{b}	130.4±8.45^{b}

B. ***Females***

	Feed Intake (g/100g body mass)	Fluid Intake (mL/100g body mass)	Calorie Intake (Kcal/100g body mass)
NM+W^{ad}	48.88±4.24^{a}	103.6±16.05^{a}	136.9±11.87^{a}
NM+Eth+W^{ad}	46.83±1.85^{a}	102.4±5.4^{a}	131.1±5.19^{a}
NM+Eth^{ad}	33.62±5.38^{b}	75.47±11.76^{b}	153.5±18.40^{ab}
NM+Eth+Eth^{ad}	33.28±2.43^{b}	78.87±8.27^{b}	154.1±8.77^{b}
NM+ZO+W^{ad}	47.09±2.24^{a}	114.7±23.71^{a}	131.8±6.27^{a}
NM+Eth+ZO+W^{ad}	47.10±2.86^{a}	101.6±14.42^{a}	131.9±7.51^{a}
NM+ZO+Eth^{ad}	33.24±1.83^{b}	79.09±11.62^{b}	153.8±12.32^{b}
NM+Eth+ZO+Eth^{ad}	33.72±2.49^{b}	77.40±13.41^{b}	154.1±8.25^{b}

Data presented as mean ± standard deviation. [ab] = within column means with different letters significantly different at $p < 0.050$. NM+Eth decreased glucose. **NM + *W*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk during suckling + plain drinking water in adulthood; **NM+Eth+ *W*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk and Eth during suckling + plain drinking water in adulthood; **NM+*Eth*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk during suckling + Eth solution in adulthood; **NM + Eth+ *Eth*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk and Eth during suckling + Eth solution in adulthood; **NM+ ZO + *W*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk and ZO during suckling + plain drinking water in adulthood; **NM+Eth+ZO+*W*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk, Eth and ZO during suckling + plain drinking water in adulthood; **NM+ZO+*Eth*^{ad}** = gavage with 10 mL/kg body mass per day nutritive milk and ZO during suckling + Eth solution in adulthood; **NM+Eth+ZO+ *Eth*^{ad}** = gavage with 10 mL/kg body mass per day nutritive milk, Eth and ZO during suckling + Eth solution in adulthood n = 6-8 per treatment group

4.4.3 Effect of neonatal orally administered zingerone on visceral fat mass

Figure 4.4 shows the visceral fat mass of male and female rats. In males, early, late single hit, double hit alcohol, and neonatal zingerone did not have an effect ($p = 0.742$; Fig 4.4A) on the visceral fat mass.

There was an overall statistical difference ($p = 0.001$; Fig 4.4B) in the visceral fat mass in female rats. The early, late single and double hit with alcohol, as well as neonatal ZO, did not have an effect ($p > 0.05$ vs control) on the visceral fat mass. However, female rats that received the combination of neonatal ZO and a late single hit with alcohol (NM+ZO+Eth^{ad}) had significantly lower ($p = 0.045$, $p = 0.007$, $p = 0.005$) visceral fat mass compared to female rats that had the control (NM+W^{ad}), early single hit alone (NM+Eth+W^{ad}) or in combination with neonatal ZO (NM+Eth+ZO+W^{ad}), respectively.

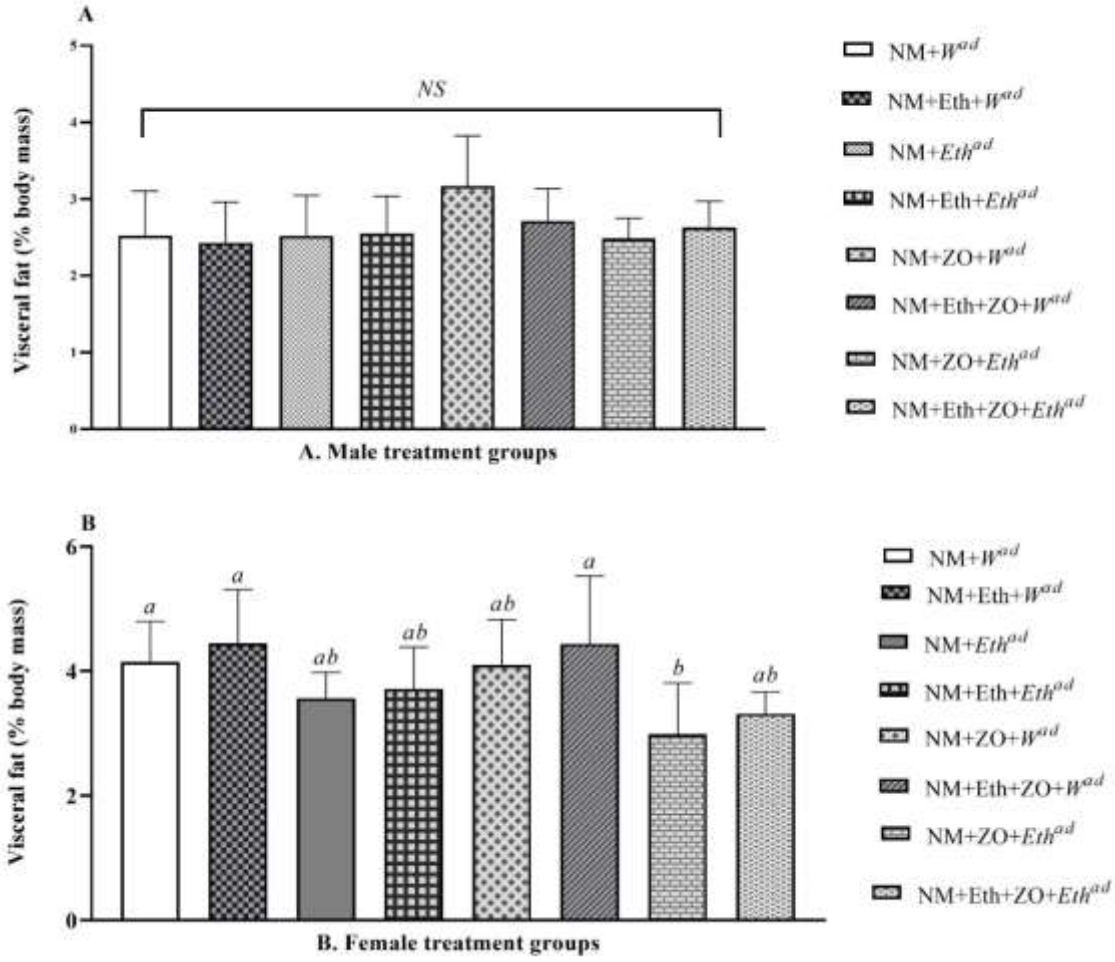

Figure 4.4:The effect of neonatal zingerone on visceral fat mass of male (A) and female (B) rats drinking alcohol in adulthood

Data presented as mean ± standard deviation. NS: No significant difference was observed across treatment groups. ^{ab} = significant difference between visceral fat mass at $p < 0.05$. **control (NM+W^{ad})** – neonatally gavaged with nutritive milk (NM) and received plain drinking water (W^{ad}) at stage III; **early single hit (NM+Eth+W^{ad})** - neonatally gavaged with ethanol in nutritive milk and received plain drinking water (W^{ad}) at stage III; **late single hit (NM+Eth^{ad})** - neonatally gavaged with NM and received ethanol (Eth^{ad}) solution at stage III; **double hit (NM+Eth+Eth^{ad})** - neonatally gavaged with Eth in nutritive milk and received Eth^{ad} at stage III; **zingerone alone (NM+ZO+W^{ad})** - neonatally gavaged with ZO in nutritive and received plain drinking water (W^{ad}) at stage III; **early single hit with zingerone (NM+Eth+ZO+W^{ad})** - neonatally gavaged with NM, Eth, ZO and received plain drinking water (W^{ad}) at stage III; **late single hit with zingerone (NM+ZO+Eth^{ad})** - neonatally gavaged with NM and ZO and

received *Eth*^{ad} at stage III; **double hit with zingerone (NM+Eth+ZO+*Eth*^{ad}) -** neonatally gavaged with NM, Eth and ZO and received *Eth*^{ad} at stage III; NM-Nutritive milk; Eth-ethanol; ZO-zingerone; W-water; n = 6-8 per treatment group

4.4.4 Effect of neonatal orally administered zingerone on blood glucose, adiponectin, insulin, leptin and HOMA-IR

Table 4.2 shows data on fasting blood glucose, adiponectin, leptin, insulin and HOMA-IR. Male rats that had a late single hit of alcohol had a significantly reduced ($p = 0.026$ vs control; Table 4.2A) blood glucose concentration. Neonatal ZO mitigated ($p = 0.096$ vs control; Table 4.2A) the reduction in glucose concentration induced by the late single hit of alcohol in male rats. Treatments had no effect ($p > 0.05$; Table 4.2B) on the blood glucose concentration of female rats. Early and late single hits, a double hit of alcohol, as well as neonatal ZO alone did not have an effect ($p > 0.05$; Table 4.2A & 4.2B) on adiponectin, insulin and leptin concentrations as well as HOMA-IR of male and female rats.

Table 4.2:Effect of neonatal orally administered zingerone on the glucose, adiponectin, leptin, insulin concentrations and HOMA-IR in male (A) and female (B) adult rats drinking alcohol in adulthood

A. Males

Treatment group	Glucose (mmol/L)	Adiponectin (ng/mL)	Leptin (ng/L)	Insulin (ng/mL)	HOMA-IR
NM+W^{ad}	4.36 ± 0.44^{a}	17.95±1.96^{a}	1.63±0.54^{a}	42.35±17.46^{a}	0.20±0.08^{a}
NM+Eth+W^{ad}	3.91 ± 0.40^{ab}	22.57±7.04^{a}	1.32±0.56^{a}	44.93±15.27^{a}	0.20±0.09^{a}
NM+Eth^{ad}	3.6 ± 0.25^{b}	21.11±8.95^{a}	1.41±0.63^{a}	58.45±16.47^{a}	0.22±0.06^{a}
NM+Eth+Eth^{ad}	3.80±0.35^{ab}	14.60±2.27^{a}	1.39±0.59^{a}	39.34±14.34^{a}	0.17±0.08^{a}
NM+ZO+W^{ad}	3.87±0.29^{ab}	18.24±4.28^{a}	2.09±0.54^{a}	49.64±11.03^{a}	0.23±0.06^{a}
NM+Eth+ZO+W^{ad}	4.03±0.55^{ab}	30.25±13.53^{a}	2.28±0.89^{a}	48.93±12.67^{a}	0.23±0.05^{a}
NM+ZO+Eth^{ad}	3.72±0.27^{ab}	17.91±4.89^{a}	1.34±0.24^{a}	44.49±20.64^{a}	0.18±0.09^{a}
NM+Eth+ZO+Eth^{ad}	3.80±0.46^{ab}	20.19±5.81^{a}	2.06±0.65^{a}	51.07±27.02^{a}	0.22±0.14^{a}

B. Females

Treatment group	Glucose (mmol/L)	Adiponectin (ng/mL)	Leptin (ng/mL)	Insulin (pg/mL)	HOMA-IR
NM+W^{ad}	3.84±0.34^{a}	24.77±3.55^{a}	1.58±0.76^{a}	40.11±14.28^{a}	0.17±0.06^{a}
NM+Eth+W^{ad}	3.91±0.53^{a}	19.68±6.61^{a}	1.99±0.74^{a}	50.60±24.97^{a}	0.24±0.13^{a}
NM+Eth^{ad}	3.73±0.32^{a}	18.61±2.96^{a}	1.23±0.42^{a}	50.40±17.99^{a}	0.21±0.08^{a}
NM+Eth+Eth^{ad}	3.70±0.33^{a}	20.04±6.38^{a}	1.74±0.49^{a}	48.66±12.26^{a}	0.19±0.06^{a}
NM+ZO+W^{ad}	3.96±0.36^{a}	22.95±3.51^{a}	1.68±0.30^{a}	45.14±11.93^{a}	0.20±0.06^{a}
NM+Eth+ZO+W^{ad}	4.04±0.56^{a}	29.49±14.04^{a}	1.44±0.47^{a}	51.98±19.86^{a}	0.24±0.12^{a}
NM+ZO+Eth^{ad}	3.76±0.46^{a}	18.56±3.96^{a}	1.61±0.48^{a}	42.99±11.15^{a}	0.18±0.04^{a}
NM+Eth+ZO+Eth^{ad}	3.58±0.27^{a}	17.68±3.95^{a}	1.18±0.23^{a}	35.11±6.88^{a}	0.14±0.05^{a}

Data presented as mean ± standard deviation. ^{ab} = within column means with different letters significantly different at $p < 0.050$. NM+Eth decreased glucose. **NM + W^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk during suckling + plain drinking water in

adulthood; **NM+Eth+ W^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk and Eth during suckling + plain drinking water in adulthood; **NM+Eth^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk during suckling + Eth solution in adulthood; **NM + Eth+ Eth^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk and Eth during suckling + Eth solution in adulthood; **NM+ ZO + W^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk and ZO during suckling + plain drinking water in adulthood; **NM+Eth+ZO+W^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk, Eth and ZO during suckling + plain drinking water in adulthood; **NM+ZO+Eth^{ad}** = gavage with 10 mL/kg body mass per day nutritive milk and ZO during suckling + Eth solution in adulthood; **NM+Eth+ZO+ Eth^{ad}** = gavage with 10 mL/kg body mass per day nutritive milk, Eth and ZO during suckling + Eth solution in adulthood n = 6-8 per treatment group

4.4.5 Effect of neonatal orally administered zingerone on plasma lipid concentration

The early, late single hit and a double hit of alcohol as well as neonatal orally administered zingerone had no effect ($p > 0.05$, all treatments vs control; Table 4.3A & 4.3B) on the plasma triglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol concentration in male and female rats. However, male rats that had a double hit of alcohol in combination with neonatal orally administered zingerone (NM+Eth+ZO+Eth^{ad}) had significantly lower ($p = 0.0473$; Table 4.3A) plasma HDL-cholesterol concentration compared to male rats that received neonatal zingerone alone.

Table 4.3: Effect of neonatal orally administered zingerone on plasma lipid concentration in male (A) and female (B) adult rats drinking alcohol in adulthood

A. Males

Treatment Group	Triglycerides (mmol/L)	Total cholesterol (mmol/L)	HDL-chol (mmol/L)	LDL-chol (mmol/L)
NM+W^{ad}	0.86±0.18^{a}	3.33±0.57^{a}	1.04±0.14^{ab}	1.99±0.70^{a}
NM+Eh+W^{ad}	0.81±0.20^{a}	3.26±0.38^{a}	0.91±0.161^{ab}	1.82±0.26^{a}
NM+Eth^{ad}	0.92±0.10^{a}	3.31±0.29^{a}	0.87±0.07^{ab}	2.00±0.26^{a}
NM+Eth+Eth^{ad}	0.77±0.13^{a}	3.02±0.30^{a}	0.83±0.14^{ab}	1.77±0.14^{a}
NM+ZO+W^{ad}	0.70±0.14^{a}	3.17±0.36^{a}	1.05±0.23^{a}	1.77±0.16^{a}
NM+Eth+ZO+W^{ad}	0.82±0.14^{a}	3.15±0.35^{a}	0.93±0.097^{ab}	1.86±0.31^{a}
NM+ZO+Eth^{ad}	0.81±0.18^{a}	3.27±0.43^{a}	0.83±0.10^{ab}	2.14±0.36^{a}
NM+Eth+ZO+Eth^{ad}	0.89±0.13^{a}	3.06±0.16^{a}	0.80±0.10^{b}	1.85±0.12^{a}

B. Females

Treatment Group	Triglycerides (mmol/L)	Total cholesterol (mmol/L)	HDL-chol (mmol/L)	LDL-chol (mmol/L)
NM+W^{ad}	0.84±0.11^{a}	1.42±0.23^{a}	3.64±0.50^{a}	1.84±0.45^{a}
NM+Eh+W^{ad}	1.02±0.30^{a}	1.32±0.27^{a}	3.51±0.40^{a}	2.01±0.431^{a}
NM+Eth^{ad}	0.87±0.13^{a}	1.29±0.20^{a}	3.30±0.61^{a}	1.63±0.56^{a}
NM+Eth+Eth^{ad}	0.79±0.14^{a}	1.45±0.20^{a}	3.78±0.45^{a}	1.73±0.43^{a}
NM+ZO+W^{ad}	0.84±0.18^{a}	1.32±0.17^{a}	3.42±0.37^{a}	1.70±0.40^{a}
NM+Eth+ZO+W^{ad}	0.76±0.17^{a}	1.45±0.12^{a}	3.37±0.47^{a}	1.58±0.47^{a}
NM+ZO+Eth^{ad}	0.91±0.22^{a}	1.46±0.23^{a}	3.64±0.40^{a}	1.77±0.27^{a}
NM+Eth+ZO+Eth^{ad}	0.78±0.16^{a}	1.23±0.30^{a}	3.22±0.20^{a}	1.63±0.31^{a}

Data presented as mean ± standard deviation. [ab] = within column means with different letters significantly different at $p < 0.05$. **NM + *W*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk during suckling + plain drinking water in adulthood; **NM+Eth+ *W*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk and Eth during suckling + plain drinking water in adulthood; **NM+*Eth*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk during suckling + Eth solution in adulthood; **NM + Eth+ *Eth*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk and Eth during suckling + Eth solution in adulthood; **NM+ ZO + *W*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk and ZO during suckling + plain drinking water in adulthood; **NM+Eth+ZO+*W*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk, Eth and ZO during suckling + plain drinking water in adulthood; **NM+ZO+*Eth*^{ad}** = gavage with 10 mL/kg body mass per day nutritive milk and ZO during suckling + Eth solution in adulthood; **NM+Eth+ZO+ *Eth*^{ad}** = gavage with 10 mL/kg body mass per day nutritive milk, Eth and ZO during suckling + Eth solution in adulthood n = 6-8 per treatment group.

4.5 Discussion

This study investigated the potential of zingerone to protect against adverse early single hit, late single hit and double hit with alcohol. We provide first-time evidence that an early and late single hit as well as double hit with alcohol did not affect body mass, insulin, leptin and adiponectin concentrations, HOMA-IR and visceral adiposity in male and female rats. However, a late single hit with alcohol in males resulted in a decreased blood glucose concentration. Neonatal orally administered zingerone mitigated the single hit alcohol-induced reduction in blood glucose concentration in male rats. A combination of neonatal zingerone and a late hit of alcohol reduced visceral fat mass in female rats.

In this study, alcohol consumption decreased fluid intake by male and female rats that were administered neonatally with either alcohol or zingerone. This is likely due to natural aversion to ethanol by rats (Lamas-Paz et al., 2018). A late single hit with alcohol had no effect on male rats' ethanol consumption. Previous studies have shown that ethanol consumption increases in males, particularly when exposed to it (ethanol) in adolescence (late single hit) (Varlinskaya et al., 2015; Towner & Varlinskaya, 2020). However, the method of ethanol administration in these studies was different from our study since these studies that reported an increase in ethanol intake due to adolescent exposure used intermittent access to the ethanol solution (Pandey et al., 2015; Sakharkar et al., 2019; Varlinskaya et al., 2015) which is known to induce escalated alcohol consumption in rodents (Carnicella et al., 2014). Other studies have also reported that adolescent ethanol exposure does not influence its consumption (Füllgrabe et al., 2007; Vetter et al., 2007). Therefore, neither neonatal orally administered alcohol nor late single hit with alcohol influenced fluid intake in this study.

As previously reported by Fromenty et al. (2009) and Kołota et al. (2019), long-term ethanol consumption in adult rats reduced feed intake, a similar finding in the current study. Rats provided with alcohol as the only drinking fluid increase pre-prandial drinking (Laure-Achagiotis et al.,1990). It is demonstrated that alcohol displaces energy from fats, carbohydrates, and protein as it becomes the preferred source of energy (Shelmet et al., 1988), partly explaining how the rats that consumed alcohol had a reduced feed intake. In the current study, eight weeks of alcohol consumption increased calorie intake. Thus, the increased calorie intake observed is likely the effect of high calorie value of alcohol. Calorie contribution from alcohol to overall calorie intake of all rats that had ethanol solution at stage III of the experiment ranged from 33.7% to 39.5%. Alcohol-induced increase in calorie intake did not affect the body masses of the rats. We noted that calorie intake increases were evident towards the end of the experimental period, hence probably did not impact body mass gain. Furthermore, this might have resulted from alcohol-induced activation of brown adipose tissue (BAT), which dissipates extra energy through non-shivering thermogenesis (Saely et al., 2012; Larue-Achagiotis et al., 1990). Alcohol consumption is associated with decrease in body temperature in the night and decrease in BAT activation (Blaner et al., 2017). However, we did not measure the body temperature of the rats during the experiment.

Despite a significant increase in calorie intake, neonatal zingerone combined with a late single alcohol hit decreased visceral fat mass in female rats in this study suggesting synergy between neonatal zingerone and later alcohol consumption at preventing development of visceral obesity. There are few alcohol studies in female rodents due to the impact of oestrous cyclicity (Beery, 2018). However, available data in male rats suggest that the effect of alcohol on visceral fat is affected by dose, pattern and mode of administration (Steiner & Lang, 2017). Consumption of a

6% ethanol solution for 28 days in adult male Wistar rats resulted in decreased visceral fat mass (Pravdova et al., 2009) via increased lipolysis and redistribution of fat to central regions (Pravdova et al., 2009; Steiner & Lang, 2017). Zingerone also protects against visceral fat mass accumulation (Muhammad et al., 2021) by increasing lipolysis and activating the peroxisome proliferator-activated receptor-γ (PPARγ) (Chung et al., 2009; Ahmad et al., 2015). PPARγ reduces visceral fat via trans-differentiation of white adipose tissue to brown adipose tissue (Kajimura et al., 2009). The combined effect of alcohol-induced lipolysis in adulthood and 'browning' of white adipose tissue and additional lipolysis by neonatal zingerone resulted in the significant visceral fat mass reduction observed in the female rats. This synergistic effect was not apparent in the male rats, suggesting sexual dimorphic responses to the interventions. We cannot explain this observation; however, we speculate that the combined effect of ethanol and zingerone was ineffective in the male rats because of android obesity (Nauli & Matin, 2019), albeit this requires further investigation. Unexpectedly, a double hit with alcohol in combination with neonatal zingerone did not cause a significant visceral fat mass reduction, implying that when administered simultaneously, alcohol reduces the potential protective effect of ZO against visceral fat accumulation.

During lipolysis adipose tissue secretes adipokines, leptin and adiponectin (Kaisanlahti & Glumoff, 2019). However, the long-term effect of alcohol consumption on leptin is unclear (Hiney et al., 1999; Maldonado-Devincci et al., 2010; Tan et al., 2012). Generally, circulating leptin levels appear unrelated to alcohol intake but rather regulates feed intake and body weight in proportion to body fat (Stern et al., 2016). Under long-term alcohol intake, leptin is not central to adipose tissue metabolism of alcohol, thus producing inconsistent results in rodents and humans (Stern et

al., 2016). Although eight weeks of alcohol consumption reduced feed intake in both sexes, body weight was not affected, suggesting no intervention affected leptin sensitivity. Previous report indicated that feed intake and body weight is not mediated by leptin under long-term alcohol consumption (Štrbák et al., 1998).

The preponderance of available data in rodent studies (late single hit in adulthood) report that long-term alcohol administration reduces ADP levels in males (Chen et al., 2007; Yu et al., 2010; Yang et al., 2017) but increases it in females (Fulham & Mandrekar, 2016; DeGroat et al., 2018). There are no comparative studies in which the effect of alcohol consumption exclusively was assessed in rats for contextual comparison except one wherein, high dose ethanol (32g/kg/day) via intragastric intubation resulted in a hyperadiponectinemia (Xu et al., 2011). Human studies also show a positive relationship between adiponectin and liver injury and inflammation in ALD patients (Buechler et al., 2009; Kasztelan-Szczerbinska et al., 2013). The administration of recombinant adiponectin has been shown to attenuate liver injury and inflammation in ALD mice models (Xu et al., 2003). However, other alcohol models show that ADP is elevated in ALD with significant inflammation (Fulham & Mandrekar, 2016; DeGroat et al., 2018). Thus, though ADP may be elevated under long-term alcohol consumption, its signalling may be impaired. Tan et al. (2012) observed an increase in ADP during the first two weeks of alcohol consumption which declined to normal levels by the 4^{th} week and remained so up until the 8^{th} week. In this study, ADP concentrations remained at control levels after eight weeks of ethanol consumption in both male and female rats therefore ADP levels had possibly returned to normal levels.

In the current study, neonatal alcohol exposure (NAE) in male and female rats did not affect insulin action and signalling; hence blood glucose concentration and the cells' sensitivity to insulin were unaffected. In agreement with other developmental studies, early-life alcohol exposure alone does

not significantly affect insulin concentration, sensitivity to insulin and fasting blood glucose concentration (Yao & Nyomba, 2008; Amos-Kroohs et al., 2018) and if at all, the effect is subtle and sex-specific affecting males more significantly than females (Probyn et al., 2013). In male rats, a late single hit with alcohol significantly decreased blood glucose concentration. Alcohol consumption decreases blood glucose concentration in male rats (Sumida et al., 2004). A previous study indicated that prenatal alcohol exposure in male SD rats caused increased gluconeogenesis and upregulated gluconeogenic genes in adult offspring (Yao et al., 2013). Hence unlike those neonatally administered with alcohol, rats in the late single hit group were not cushioned against glucose depletion. However, gluconeogenesis was not completely uninhibited due to the modest increase in insulin. A modest increase in insulin promotes lipogenesis and inhibits lipolysis (Kolb et al., 2020) partially explaining the non-reduction in visceral fat mass accumulation. A decrease in blood glucose concentration was not evident in female rats that received a late single hit with alcohol primarily because females are more insulin sensitive than males (Tramunt et al., 2020). Oral administration of zingerone did not affect insulin and hence glucose metabolism. Other studies report that zingerone has an anti-diabetic effect (Cui et al., 2018; Ahmad et al., 2018; Anwer et al., 2019) possibly due to its potential to mediate decreased and increased gluconeogenesis and glycogenolysis, respectively (Otunola & Afolayan, 2019). This partly explains how neonatal orally administered zingerone may have mitigated decreased blood glucose concentration in rats that had ethanol solution (alcohol) as a drinking fluid in adulthood.

Long-term alcohol consumption, especially in the fasting state leads to lipolysis and a release of free fatty acids into circulation (Wei et al., 2013). However, we observed no significant difference in plasma total cholesterol, triglycerides, HDL- and LDL-cholesterol of adult rats regardless of intervention. Chen & Nyomba, (2004) reported that lactational alcohol exposure causes

dyslipidaemia in adult offspring but only in those that experienced growth-restriction. Since the rats in our study did not experience growth restriction, our findings study collaborate those reported by Chen and Nyomba (2004). In other studies, male adult Wistar rats on long-term alcohol showed a decrease in plasma triglycerides (Radic et al., 2018) or increased triglycerides (Ojeda et al., 2008) at ethanol consumption level of 8.14g/kg bw/day and 15.9g/kg bw/day, respectively. The discrepancy between our findings with their results could be due to rat-strain dependent differences in the efficiency of ethanol metabolism (DeNucci et al., 2010) and the difference in the amount of ethanol consumed by the rats. Furthermore, free fatty acids can be taken up by the liver and esterified to form triglycerides leading to fatty liver formation (Rasineni et al., 2019). Therefore, organs such as the liver, kidney and skeletal muscle might have mopped up the plasma lipids (Steiner & Lang, 2017) resulting in no significant changes in plasma triglycerides observed in the current study. Nonetheless, a recent study indicates that zingerone protects against hypertriglyceridemia in Sprague-Dawley rats (Muhammad et al., 2021) by mediating its anti-hyperlipidaemic property through stimulation of increased lipolysis and upregulation of noradrenaline-sensitive lipases (Ahmad et al. 2015).

Alcohol has a J-shape relationship with metabolic outcomes (Yun et al., 2021). Hence, it was surprising that though the model employed achieved a moderate to high ethanol intake [>5g/kg/day (Leeman et al., 2010)] marked metabolic effects were not evident. This maybe because of the fast metabolic rate of ethanol metabolism and elimination in rats (Lamas-Paz, Hao, Nelson, Vázquez, Canals, Moral, et al., 2018). In tandem with our findings other studies have also found that ethanol consumption in rats had no significant impact on metabolic outcome measures (Štrbák et al., 1998; Radic, Nestorović, et al., 2018; Justice et al., 2019; Yamasaki et al., 2019). Additionally, the age

of the rats might be a contributory factor. Compared to aged (≥24months) rats that were used in other studies (Gårdebjer et al., 2015; Xiao et al., 2019), we and other researchers (Amos-Kroohs et al., 2018; Radic et al., 2018; Kołota, 2018) show that young adult rats are resistant to cardio-metabolic effects of alcohol.

4.6 Conclusion

In summary, although NAE or adult alcohol consumption did not cause marked metabolic effects, neonatal orally administered zingerone created a favourable physiological state that prevented the alcohol-induced decrease in blood glucose concentration in males only and decreased visceral fat accumulation in female rats. Therefore, zingerone, when administered during the suckling growth phase, can programme for some protection against metabolic consequences of chronic alcohol consumption in adulthood. It can therefore be considered as a preventive strategy in the form of a dietary supplement in the fight against metabolic derangements induced by alcohol consumption.

The next chapter is made up of a published paper where the potential of neonatal orally administered zingerone to programme for protection against alcohol-indiced liver fatty liver disease in adulthood was explored. Unlike in the previous chapter where potetnial to programme for protection against generalised alcohol-induced metabolic derangments, the published paper which makes up chapter 5 gives a specific narrative of zingerone's potential to programme for protection against alcohol-indcued fatty liver disease following a double-hit with alcohol.

4.7 References

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CHAPTER FIVE-NEONATAL ORALLY ADMINISTERED ZINGERONE ATTENUATES ALCOHOL-INDUCED FATTY LIVER DISEASE IN EXPERIMENTAL RAT MODELS

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5.0 Abstract

Alcohol intake at different developmental stages can lead to the development of alcohol-induced fatty liver disease (AFLD). Zingerone (ZO) possess hepatoprotective properties, thus when administered neonatally it could render protection against AFLD. This study aimed to evaluate the potential long-term protective effect of ZO against the development of AFLD. One hundred and twenty-three 10-day-old Sprague-Dawley rat pups (60 males; 63 females) were randomly assigned to four groups and orally administered the following treatment regimens daily during the pre-weaning period from postnatal day (PND) 12-21: group 1 - nutritive milk (NM), group 2 - NM +1 g/kg ethanol (Eth), group 3 - NM + 40 mg/kg ZO, group 4 - NM + Eth +ZO. The rats were weaned at PND 21 and then grown to PND 45 on a standard rat chow and plain drinking water. From PND 46-100, each group from the neonatal stage was divided into two where subgroup I had tap water and subgroup II had ethanol solution as drinking fluid, respectively, for eight weeks. Ethanol intake, liver morphometry and fat content, plasma alanine transaminase and aspartate transaminase (ALT and AST) activities, tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), cytochrome P4502E1 (CYP2E1) concentrations and hepatic lipid peroxidation were measured. Hepatic mRNA expressions of peroxisome proliferator activator receptor-alpha (*PPAR-α*), sterol regulatory

element binding protein 1c (*SREBP1c*), nuclear factor kappa beta (*NF-Kβ*) and *TNF-α* were analysed by quantitative reverse transcriptase polymerase chain reaction.

Mean daily ethanol intake, which ranged from 10 to 14.5g/kg body mass/day, resulted in significant CYP2E1 elevation in male and female rats versus control ($p < 0.05$). Both late single hit and double hit with alcohol increased liver fat content, caused hepatic macrosteatosis, upregulated mRNA expression of *SREBP1c* but downregulated *PPAR-α* in male and female rats ($p < 0.05$). However, neonatal orally administered ZO protected against liver lipid accretion and *SREBP1c* upregulation in male rats only and attenuated the alcohol-induced hepatic *PPAR-α* downregulation and macrosteatosis in both sexes. This data suggests that neonatal orally administered zingerone can be a potential prophylactic agent against the development of AFLD.

Keywords: alcohol-induced fatty liver disease, zingerone, peroxisome proliferator activator receptor-alpha (*PPAR-α*), sterol regulatory element binding protein 1c (*SREBP1c*), macrosteatosis

5.1 Introduction

Alcohol liver disease (ALD) develops from prolonged excessive alcohol consumption. Studies show that genetic predisposition contributes substantially to alcohol use disorder (Tawa et al., 2016; Siomek-Gorecka et al., 2021). Others show that exposure to alcohol *in utero* trigger epigenetic modifications that also may play a role in mediating alcohol use disorders (Nizhnikov et al., 2014; Meroni et al., 2018). Numerous studies indicate that the intrauterine environment can set either a healthy or diseased trajectory for offspring in the future; a phenomenon described as the developmental origin of health and disease (Gluckman et al., 2010; Ellis et al., 2014). We hypothesized that ALD may develop based on this concept (Asiedu et al., 2021). Human and

preclinical studies indicate that prenatal alcohol exposure (PAE) predisposes offspring to increased alcohol consumption in adolescence and adulthood (Hannigan et al., 2015; Gaztañaga et al., 2020). Consumption of alcohol in adolescence has been shown to cause use disorders and abuse, which may result in the development of ALD (Rangmar et al., 2015; Hagström et al., 2018). Hence, early-life exposure to alcohol and/or alcohol consumption in adolescence may be a significant risk factor for ALD. Therefore, the combination of early-life alcohol exposure and alcohol consumption in adolescence constitute a double-hit effect for the development of ALD. Although the suckling growth phase is equally susceptible to neuroplasticity as the prenatal phase (Ellsworth et al., 2018), its impact on alcohol consumption in adolescence and the subsequent development of ALD have not been thoroughly explored.

Chronic liver disease affects about 10% of the world's human population and its mortal end-stage generally follows cirrhosis and liver cancer (Muriel, 2017). Varied factors characterize liver disease as the fourth to the fifth cause of death worldwide (Muriel, 2017). Non-alcoholic fatty liver disease ranks first contributing up to 40% of liver diseases and hepatitis B and C viruses and alcohol overconsumption contribute 30%, 15% and 11%, respectively (Muriel, 2017). Alcohol abuse ranks third to smoking and hypertension as a cause of preventable death (Singal & Anand, 2013). Alcohol-induced liver disease accounts for 20% to 50% of the prevalence of liver cirrhosis (WHO, 2018). Alcohol consumption results in liver damage on a spectrum; from simple steatosis to hepatitis with fibrosis (Gao & Bataller, 2011; Teschke, 2018). The development of a fatty liver is the earliest response to heavy (>40g ethanol/day) and/or chronic consumption of alcohol and this occurs in about 90% of heavy drinkers (Ohasie et al., 2018). However, only 8-20% of those that develop fatty liver progress to severe forms of liver disease (Ohasie et al., 2018).

Steatosis, which is categorized as either macro- and or micro-steatosis, occurs when liver tissue constitutes more than 5% fat resulting in hepatocytes becoming distended with lipids (Nassir et al., 2015). Hepatic macrosteatosis is typified by the presence of a large fat droplet that pushes the nucleus to the periphery of the hepatocyte (Yeh & Brunt, 2014; Nassir et al., 2015) while with microsteatosis many small, less than 1 μm in diameter, cytoplasmic lipid droplets give the hepatocyte a foamy-like appearance but with the nucleus remaining in the middle of the cell (Yeh & Brunt, 2014; Nassir et al., 2015). There are two variants of macrosteatosis; large and small droplet macrosteatosis (Mostafa et al., 2020). In the former, a large unilocular lipid droplet fills up the hepatocyte and pushes the nucleus to the periphery and in the latter are multilocular lipid droplets that occupy less than half of the cytoplasm with the nucleus remaining intact (Mostafa et al., 2020). In the progression of steatosis, small lipid droplets coalesce into large fat droplets (Thiese et al., 2013). Studies show that macrosteatosis is common (Sakhuja, 2014) but microsteatosis is rare in AFLD (Theise, 2013). Macrosteatosis has a good prognosis with rare progression to fibrosis (Tandra et al., 2011).

Although the pathogenesis of ALD is not entirely understood, it is known to stem from the toxic effects of ethanol and its metabolite acetaldehyde (AA), which mediate increased intestinal permeability, changes to the gut microbiome and an inflammatory response to cellular injury (Dunn and Shah, 2016). *In vivo*, alcohol dehydrogenase (ADH) metabolises ethanol to AA. The metabolism of ethanol involves the upregulation of sterol regulatory element-binding transcription factor 1 c (*SREBP-1c*), which on interacting with lipid droplets membrane proteins, promotes the formation of lipid droplets in hepatocytes resulting in the development of steatosis (Gu et al., 2015). An increase in the reduced nicotinamide adenine dinucleotide (NADH) to oxidised nicotinamide adenine dinucleotide (NAD^+) ratio inhibits β-oxidation and activates triglyceride

synthesis (Dunn & Shah, 2016), which increases the quantity of fat in hepatocytes. Additionally, the inhibition of the transactivation activity and DNA-binding of peroxisome activator receptor α (PPAR-α) by AA results in inhibiting β-oxidation (Gu et al., 2015). The metabolism of low to moderate amounts of consumed alcohol induces the ADH system but when excess alcohol is consumed, its metabolism induces the cytochrome P450 E21 (CYP2E1) system (Osna et al., 2017). Induction of the CYP2E1 generates excessive amounts of reactive oxygen species that then mediate lipid peroxidation (Stickel et al., 2017). The by-products of lipid peroxidation, for example, malondialdehyde and 4-hydroxynonenal activate adaptive immunity (Dunn and Shah, 2016) and the intrinsic apoptotic pathways (Deaciuc et al., 1995). Activation of these pathways result in hepatic neutrophil infiltration and liver inflammation via the myeloid differentiation primary response gene 88 (MyD88) independent signalling pathway (Dun and Shah, 2016). Recruitment of the MyD88 mediates the activation of the pro-inflammatory transcription factor, nuclear factor kappa β (NF-Kβ) and its downstream inflammatory cytokines; tumour necrosis factor-alpha (TNF-α), Interleukin (IL)-6, IL-10 and IL-1β (Petrasek et al., 2013; Bessone et al., 2019) that contribute to hepatocellular damage.

Several pharmacological agents have proven effective as potential prophylaxes in managing adverse effects induced by exposure to toxic substances during periods of developmental plasticity (Nyakudya et al., 2018; Mohammed, 2022; Ajah et al., 2022). Zingerone has been reported to possess anti-steatotic properties that can protect against the development of fatty liver via the activation of AMP-activated protein kinase (Muhammad et al., 2021; Mohammed, 2022). Furthermore, oral supplementation of 40 mg/kg body wt of zingerone via intragastric intubation to alcohol-fed Wistar rats protected against hepatoxicity (Mani et al., 2016). We, therefore,

determined the prophylactic protective potential of zingerone against the development of alcohol-induced fatty liver disease using rat models.

5.2 Methods and materials

5.2.1 Study setting and animal use ethical clearance

This study was conducted at the Wits Research Animal Facility (WRAF) of the University of the Witwatersrand, Johannesburg, South Africa. This study complied with accepted laboratory animal use and care stipulated in the South African National standard (SANS: 10386:2008) and the animal protection act 1962, Act No. 71. Ethical clearance for the experiment (ethical clearance certificate number: 2019/10/57B) was granted by the Animal Research Ethics Committee of the University of the Witwatersrand.

5.2.2 Animals and Animal Management

The study used one hundred and twenty-three 10-day-old suckling male and female Sprague-Dawley rat pups (60 males; 63 females) from dams with 8 to 12 rat pups. During the suckling period, from postnatal day (PND) 1 to 21, the rat litter were housed with their respective dams in acrylic cages at the WRAF. At weaning from PND 21-100, the rat pups were each individually housed in acrylic cages and allowed *ad libitum* access to feed and tap water. The room temperature was maintained at 24 ± 2˚C. A 12:12 h dark-and-light cycle (with lights on at 07:00hr) was kept throughout the experimental period.

5.2.3 Experimental design

This interventional study comprised of three stages (Figure 5.1): intervention during the neonatal growth phase, a growing out phase with no intervention, and intervention during the adult growth phase (Figure 5.1). At the neonatal stage, 123 10-day-old rat pups (60 males; 63 females) following a 2-day habituation period were then randomly assigned to four groups and administered different treatment regimens as follows: Group I: Nutritive milk (NM); Group II: NM+1 g/kg body mass ethanol (NM+Eth); Group III: NM+ 40 mg/kg body mass of ZO (NM+ZO); Group IV: NM+Eth+ZO. Interventions during the first stage were administered from PND 12-21. Ethanol and zingerone dosage have previously been used in rat pups (Cheslock et al., 2000; Muhammad et al., 2021; Asiedu et al., 2022). Nutritive milk was used as the vehicle for administration of the interventions. During the second stage (PND 22-45), the weaned rats were allowed to grow to adulthood without any intervention but had *ad libitum* access to normal rat chow and plain drinking water.

The intervention during adulthood started from PND 46 and continued to PND 100. During this intervention, rats from each group at the neonatal stage were divided into two subgroups; rats in subgroup I had plain drinking water and their counterparts in subgroup II had ethanol as drinking fluid for eight weeks (Figure 5.1). The rats were adapted to incremental ethanol solution initially at 5% (v/v) for one week; then 10% (v/v) for another week, and 20% (v/v) ethanol solution for the remaining six weeks as per the protocol described by Ojeda et al. (2008). Rats have a natural aversion to alcohol and prefer ethanol solutions at lower concentrations (Aguiar & Da-Silva, 2004). Hence, they reduce their intake volumes as the ethanol concentration increases (Aguiar & Da-Silva, 2004). Thus, incremental ethanol at 5 and 10% was used to prime them to the taste of alcohol and prevent decreased intake at 20% ethanol concentration. The amount of ethanol

consumed weekly by each was measured and computed in g/100g body mass using the 'Tables for determining gram values of ethanol solution' previously described (Veale and Myers et al., 1968). The rats that were orally gavaged with alcohol during the neonatal growth phase and had ethanol solution as drinking fluid in adulthood had a double hit with alcohol, and those that were gavaged with alcohol during the neonatal growth phase only had an early single hit with alcohol. The rats in the subgroups that received alcohol solution as a drinking fluid only during adulthood had a late single hit.

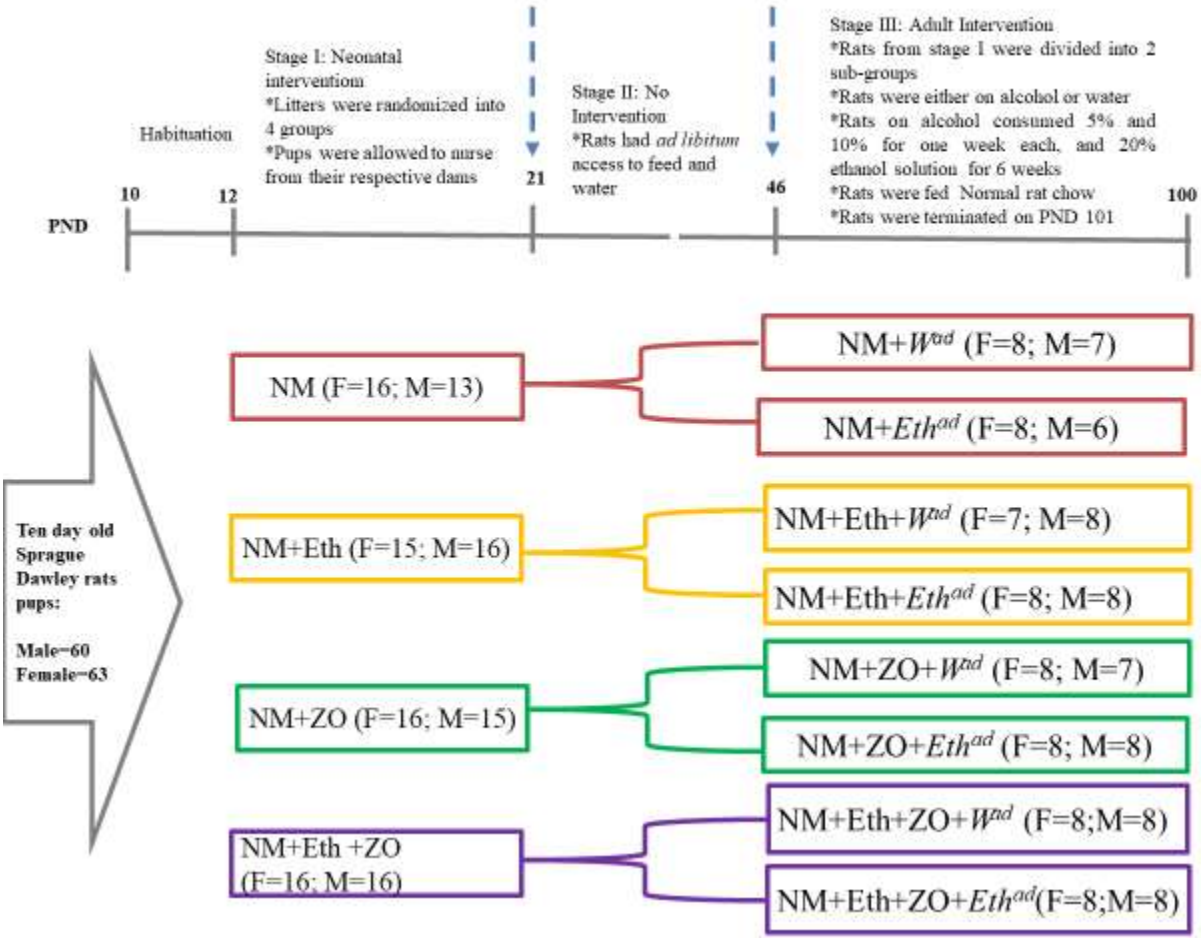

Figure 5.1:Study experimental design: **Control (NM+W^{ad})** – neonatally gavaged with nutritive milk (NM) and drank plain drinking water (W^{ad}) at stage III; **late single hit (NM+Eth^{ad})** - neonatally gavaged with NM and drank ethanol (Eth^{ad}) solution at stage III; **early single hit (NM+Eth+W^{ad}) -** neonatally gavaged with ethanol in nutritive milk and drank plain drinking water (W^{ad}) at stage III; **double hit (NM+Eth+Eth^{ad}) -** neonatally gavaged with Eth in nutritive milk and drank Eth^{ad} at stage III; **zingerone alone (NM+ZO+W^{ad})** - neonatally gavaged with ZO in nutritive and drank plain drinking water (W^{ad}) at stage III; **early single hit with zingerone (NM+Eth+ZO+W^{ad}) -** neonatally gavaged with NM, Eth, ZO and drank plain drinking water (W^{ad}) at stage III; **late single hit with zingerone (NM+ZO+Eth^{ad}) -** neonatally gavaged with NM and ZO and drank Eth^{ad} at stage III; **double hit with zingerone (NM+Eth+ZO+Eth^{ad})** - neonatally gavaged with NM, Eth and ZO and drank Eth^{ad} at stage III; F-Female; M-Male; NM-Nutritive milk; Eth-ethanol; ZO-zingerone; W-water; n = 6-8 per treatment group

5.2.4 Terminal procedure

On PND 101, following an overnight fast, the terminal body masses of the rats were measured using an electronic balance (Snowrex, Johannesburg, South Africa). The rats were then euthanised with 200 mg/kg of sodium pentobarbital (Eutha-naze®, Bayer, Johannesburg, South Africa) via intraperitoneal injection and cut open by a midline incision on the abdomen and thorax. Blood was drawn via cardiac puncture using an 18 G syringe into heparinised tubes and then centrifuged (Senova NovaFuge centrifuge, Shanghai, China) at 3000 × *g* for 15 mins. Plasma was then collected and stored at -80˚C for further biochemical assays.

Each rat's liver was carefully dissected out from the abdominal cavity and weighed on an electronic scale (Presica 310 M, Presica Instruments, Dietikon, Switzerland). The liver was then divided into four parts. One part was rinsed in cold saline and stored at -20˚C to determine hepatic thiobarbituric acid (TBARS). A sample from the right lobe of each liver was immersion fixed in 10% phosphate-buffered formalin solution (Merck, Johannesburg, South Africa) for histological analysis. Another part of the liver was stored in sealed ziplock plastic bags at -20˚C for total fat content determination, and the last portion was placed in RNA*later* solution and kept at -80˚C for molecular analysis.

5.2.5 Computation of the hepatosomatic index

The hepatosomatic index was computed by dividing the mass of each liver by the respective terminal body mass of each rat and expressed as a percentage (%).

5.2.6 Determination of hepatic lipid peroxidation

5.2.6.1 Liver tissue homogenisation

The liver sample (100 mg) was homogenised in 10 mL of phosphate buffer (0.1 M, pH = 7.4) with an ultra turrax homogeniser (T-25 basic, Janke & Kunkel Ultra Turrax, Germany). The resultant homogenate was centrifuged at 3 000 × *g* for 15 mins at 4°C. The resultant supernatant was used to determine hepatic lipid peroxidation by measuring the supernatant's TBARS concentration.

5.2.6.2 Determination of peroxidation in the liver

The liver TBARS concentration was estimated using the method described by Niehaus and Samuelsson (1968). Briefly, 2 mL of the supernatant from the liver sample homogenate was

diluted with distilled water in a 1:1 ratio. 2.0 mL of the working reagent [thiobarbituric acid (TBA)-trichloroacetic acid (TCA)-hydrochloric acid (HCL)] in a ratio of 1:1:1) was then added to the diluted supernatant. The mixture was boiled in a water bath for 15 mins, allowed to cool on ice for 5 mins, and then centrifuged (Senova NovaFuge centrifuge, Shanghai, China) at 3000 × g for 5 mins at room temperature. The absorbance of the supernatant obtained was read on a spectrophotometer (Beckman Coulter, California, USA) at 532 nm.

5.2.7 Determination of liver lipid content

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

5.2.8 Determination of liver histomorphometry

Liver tissue samples preserved in 10% phosphate-buffered formalin were processed with an automated tissue processor (Microm STP 120 Thermo Scientific, MA, USA), embedded in paraffin wax and sectioned at 3 µm using a microtome (Leica instruments GmbH, (Pty) Ltd, Wetzlar, Germany) for histological analysis. The sections were stained with haematoxylin and eosin (H&E) using a Gemini AS slide stainer coupled to a Clearvue cover slipper (Fisher Scientific, MA, USA). The stained liver sections were viewed under an Olympus BH2-RFCA microscope (Olympus Corporation, Tokyo, Japan) coupled to an Olympus XC 10 HD camera (Olympus Corporation, Tokyo, Japan) for histological assessment and image capture.

5.2.9 Determination of surrogate plasma biomarkers of liver function

Plasma activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using an automated clinical chemistry analyser (IDEXX VetTest® Clinical Chemistry

Analyser, IDEXX Laboratories Inc., City, USA) according to the manufacturer's instructions. The calibrated autoanalyser performed the analysis on pre-loaded disks for AST and ALT using 10 µL of plasma.

5.2.10 Determination of plasma CYP2E1, TNF-α and IL-6 concentration

Rat-specific ELISA kits (Elabscience®, Rat CYP2E1, TNF-α, IL-6 ELISA kit, Wuhan, Hubei Province, China) were used to determine the plasma CYP2E1, tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) concentrations following the manufacturers' instructions. The tests employed a sandwich ELISA principle. The optical density of the resulting reactions was measured at 450 nm on a microplate reader (Thermo Fisher Scientific Inc, City, Finland), and the sample concentrations were extrapolated from the standard curve.

5.2.11 Determination of hepatic gene expression

5.2 11. 1 RNA extraction and cDNA synthesis

Liver tissue (50 mg) was finely ground with a mortar and pestle, and the RNA was extracted using Aurum™ Total RNA Mini Kit (BioRad, California, USA). The RNA quantity was assessed by measuring the absorption at 260 nm and the purity by the 260/280 nm absorbance ratio using the NanoDrop lite spectrophotometer (Thermofisher Scientific, Johannesburg, South Africa). The volume of RNA needed to make a final concentration of 0.5 µg was calculated, and synthesized to complementary DNA (cDNA) with LunaScript supermix (Inqaba biotec, Johannesburg, South Africa). Nuclease-free water was added to make a final volume of 20 µL. The preparation was gently mixed and incubated at 25˚C for 2 mins, 55˚C for 10 mins, and 95˚C for 1 min on the

thermal cycler (PxE0.2, Thermo Fisher Scientific, Waltham, MA, USA). The cDNA samples were then stored at -20˚C until further use.

5.2.11.2 Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Real-time PCR was performed using LunaScript master mix (Inqaba biotec, Johannesburg, South) with the master mix and primers mixed according to the manufacturer's protocol. The primers used are provided in Supplementary Table S2. Complementary DNA was diluted to 1: 20 ratio with nuclease-free water. The prepared mix was added to appropriate wells in 96-well plates (Roche, Johannesburg, South Africa). The cDNA template was added last and the plate were sealed with optical adhesive covers. Quantitative real-time PCR (qRT-PCR) was measured on the LightCycler 96 (Roche diagnostics, Basel, Switzerland) following thermal cycling conditions of the manufacturers' protocol. Relative gene expression was analysed using the $-2^{\Delta\Delta CT}$ method. Gene expression was normalised to the mRNA expression of *beta-actin*.

5.3 Statistical analysis

GraphPad Prism 8 software (Graph-Pad Software Inc., San Diego, USA) was used to analyse data. Data are expressed as mean ± standard deviation. Data normality was assessed by Kolmogorov-smirnov and the shipiro tests A one-way ANOVA was used to analyse multiple-group data, followed by mean comparison using the Tukey *post hoc* test for parametric data. The Kruskal-Wallis test was used to analyse multiple group non-parametric data, followed by mean comparisons by the Dunns *post hoc* test for non-parametric. Statistical significance was considered when $p < 0.050$.

5.4 Results

5.4.1 Effect of neonatal orally administered zingerone on ethanol consumption in adult rats

Figure 5.1 shows the weekly mean ethanol intake of the rats. In male and female rats, early and late single hit as well as double hit of alcohol alone or together with neonatal zingerone did not affect ethanol consumption in adulthood ($p > 0.05$).

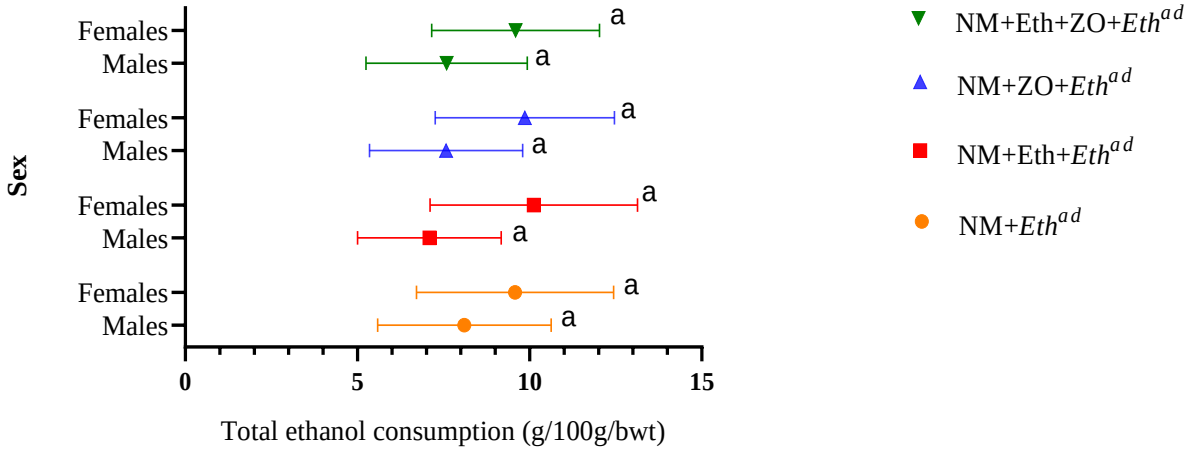

Figure 5.2: Effect of neonatal orally administered zingerone on ethanol consumption.

Data are presented as Mean ± SD. [aa] = Line graphs with similar letters do not differ significantly at $p > 0.05$. **Control (NM+W^{ad})** – neonatally gavaged with nutritive milk (NM) and drank plain drinking water (W^{ad}) at stage III; **late single hit (NM+Eth^{ad})** - neonatally gavaged with NM and drank ethanol (Eth^{ad}) solution at stage III; **early single hit (NM+Eth+W^{ad})** - neonatally gavaged with ethanol in nutritive milk and drank plain drinking water (W^{ad}) at stage III; **double hit (NM+Eth+Eth^{ad})** - neonatally gavaged with Eth in nutritive milk and drank Eth^{ad} at stage III; **zingerone alone (NM+ZO+W^{ad})** - neonatally gavaged with ZO in nutritive and drank plain drinking water (W^{ad}) at stage III; **early single hit with zingerone (NM+Eth+ZO+W^{ad})** -

neonatally gavaged with NM, Eth, ZO and drank plain drinking water (W^{ad}) at stage III; **late single hit with zingerone (NM+ZO+*Eth*^{ad})** - neonatally gavaged with NM and ZO and drank *Eth*^{ad} at stage III; **double hit with zingerone (NM+Eth+ZO+*Eth*^{ad})** - neonatally gavaged with NM, Eth and ZO and drank *Eth*^{ad} at stage III; NM-Nutritive milk; Eth-ethanol; ZO-zingerone; W-water; ^{ad}-adult treatment; n = 6-8 per treatment group

5.4.2 Effect of neonatal orally administered zingerone on hepatosomatic index

Treatment regimens had no effect on the absolute liver masses and hepatosomatic indices of the male rats ($p > 0.05$ vs control; Table 5.1). The absolute liver mass and hepatosomatic indices of the female rats in each treatment group did not differ from the control counterparts ($p > 0.05$ vs control; Table 5.1). Female rats that had an early single hit with alcohol had a significantly increased absolute liver mass compared to counterparts that had zingerone orally administered during the neonatal growth phase in combination with either a late single hit and or a double hit with alcohol [$p = 0.023$, NM+Eth+W^{ad} vs NM+ZO+*Eth*^{ad}; $p = 0.049$, NM+Eth+W^{ad} vs NM+Eth+ZO+*Eth*^{ad}]. When adjusted to the body mass, the hepatosomatic index of the female rats was not significantly different ($p > 0.05$) compared to the other treatment groups.

Table 5.1: Effect of neonatal orally administered zingerone on absolute liver mass and hepatosomatic indices in male and female rats drinking alcohol in adulthood

Treatment group	Males		Females	
	Liver mass (g)	**Hepatosomatic index (%)**	**Liver mass (g)**	**Hepatosomatic index (%)**
NM+W^{ad}	14.19±1.76^{a}	2.99±0.24^{a}	8.06±0.49^{ab}	2.92±0.10^{a}

NM+*Eth*^{ad}	13.12±1.21^{a}	2.96±0.18^{a}	7.73±0.62^{ab}	2.78±0.12^{a}
NM+Eth+*W*^{ad}	13.85±2.39^{a}	2.97±0.25^{a}	8.46±0.95^{a}	2.90±0.18^{a}
NM+Eth+*Eth*^{ad}	12.76±1.57^{a}	2.84±0.16^{a}	7.56±0.61^{ab}	2.80±0.21^{a}
NM+ZO+*W*^{ad}	14.19±1.76^{a}	2.97±0.13^{a}	7.81±0.98^{ab}	2.82±0.17^{a}
NM+Eth+ZO+*W*^{ad}	13.70±1.29^{a}	2.94±0.12^{a}	8.29±0.48^{ab}	2.89±0.18^{a}
NM+ZO+*Eth*^{ad}	11.73±1.25^{a}	2.77±0.21^{a}	7.28±0.52^{b}	2.75±0.11^{a}
NM+Eth+ZO+*Eth*^{ad}	12.30±0.95^{a}	2.80±0.19^{a}	7.38±0.41^{b}	2.82±0.10^{a}

Data are presented as Mean ± SD. ^{ab} = within column means with different letters significantly different at $p < 0.05$. **Control (NM+*W*^{ad})** – neonatally gavaged with nutritive milk (NM) and drank plain drinking water (*W*^{ad}) at stage III; **late single hit (NM+*Eth*^{ad})** - neonatally gavaged with NM and drank ethanol (*Eth*^{ad}) solution at stage III; **early single hit (NM+Eth+*W*^{ad})** - neonatally gavaged with ethanol in nutritive milk and drank plain drinking water (*W*^{ad}) at stage III; **double hit (NM+Eth+*Eth*^{ad})** - neonatally gavaged with Eth in nutritive milk and drank *Eth*^{ad} at stage III; **zingerone alone (NM+ZO+*W*^{ad})** - neonatally gavaged with ZO in nutritive and drank plain drinking water (*W*^{ad}) at stage III; **early single hit with zingerone (NM+Eth+ZO+*W*^{ad})** - neonatally gavaged with NM, Eth, ZO and drank plain drinking water (*W*^{ad}) at stage III; **late single hit with zingerone (NM+ZO+*Eth*^{ad})** - neonatally gavaged with NM and ZO and drank *Eth*^{ad} at stage III; **double hit with zingerone (NM+Eth+ZO+*Eth*^{ad})** - neonatally gavaged with NM, Eth and ZO and drank *Eth*^{ad} at stage III; ^{ad}-adult treatment NM-Nutritive milk; Eth-ethanol; ZO-zingerone; W-water; n = 6-8 per treatment group

5.4.3 Effect of neonatal orally administered zingerone on liver fat content

Treatment regimens significantly affected the liver fat content of male and female rats [p = 0.006, (males): p = 0.008, (females) Figure 5.2]. A late single hit with alcohol significantly increased (p = 0.039; Figure 5.2A) liver fat content of male rats compared to control counterparts but a combination of neonatal orally administered zingerone with a late single hit decreased liver fat content compared to male counterparts that only had a late single alcohol hit (p = 0.036, NM+ZO+Eth^{ad} vs NM+Eth^{ad}, Figure 5.2A). An early single and double hit with alcohol alone or together with neonatal administered zingerone had no effect on liver fat content of male rats (p > 0.05; Figure 5.2A). A late single and double hit with alcohol significantly increased liver fat content of female rats [p = 0.045, NM+Eth^{ad} vs NM+W^{ad}; p = 0.023, NM+Eth+Eth^{ad} vs NM+W^{ad}, Figure 5.2D]. Neonatal orally administered zingerone in combination with either a single and or double alcohol hit resulted in similar liver fat content to that of control counterparts [p = 0.858, NM+ZO+Eth^{ad} vs NM+W^{ad}; p = 0.067, NM+Eth+ZO+Eth^{ad} vs NM+W^{ad}; Figure 5.2D). The liver fat content of female rats that had a combination of neonatal zingerone and double alcohol hit was similar to that of counterparts that had a late single or double alcohol hit (p > 0.05, Figure 5.3D).

5.4.4 Effect of neonatal orally administered zingerone on hepatic histomorphometric changes

Hepatic lobules of male and female rats that consumed plain tap water in adulthood (NM+W^{ad}, NM+Eth+W^{ad}, NM+ZO+W^{ad}, NM+Eth+ZO+W^{ad}, NM+ZO+W^{ad}) contained regularly arranged hepatocytes radiating from the central vein and had no visible hepatic steatosis in the control rats (NM+W^{ad}). An early single hit with alcohol (NM+Eth+W^{ad}) resulted in visible microsteatosis in female rats (Figure 5.4; arrow D) which was not observed in the female rats that had an early single hit alcohol in combination with neonatal zingerone (Fig 5.4B). The late single hit (NM+ Eth^{ad}) and

double hit (NM+Eth+*Eth*^{ad}) with alcohol resulted in small and large droplet steatosis (Fig 5.3 & 5.4; arrow A & B) in male and female rats. A combination of neonatal zingerone with the late single hit (NM+ZO+*Eth*^{ad}) and double hit (NM+Eth+ZO+*Eth*^{ad}) of alcohol resulted in relatively less severe small and large droplet steatosis (Fig 5.3 & 5.4).

A: Males

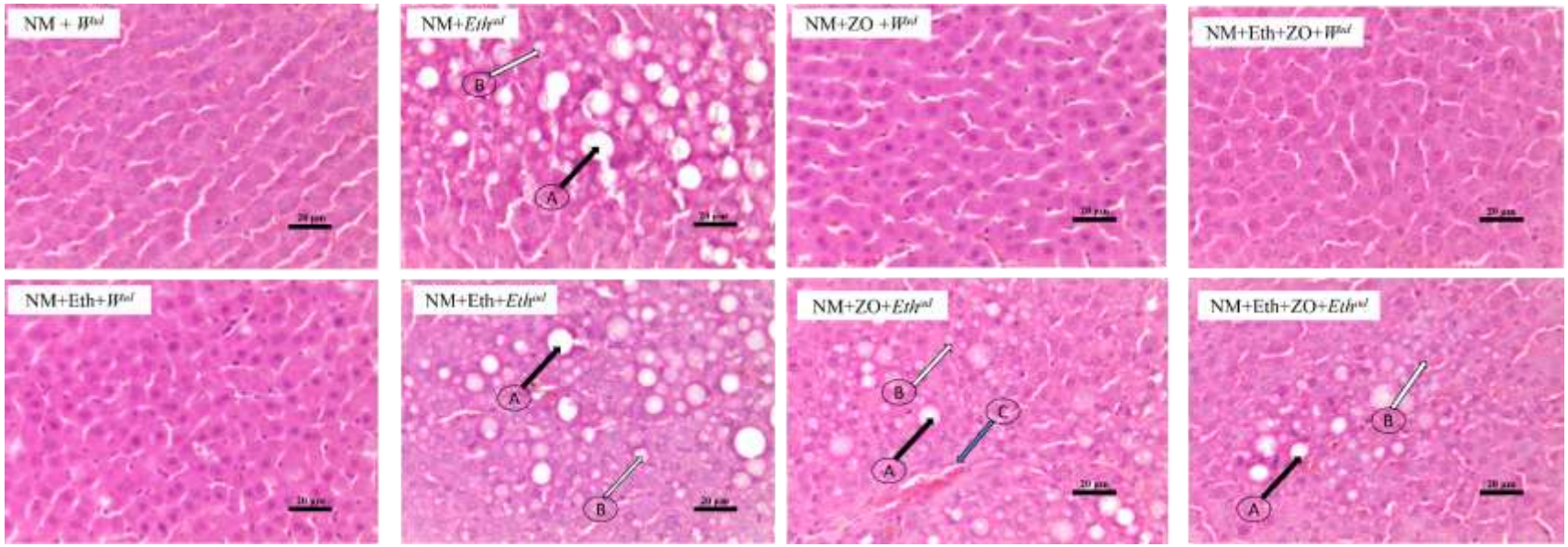

Figure 5.3: Photomicrographs of male (**A**) liver sections stained with H&E. Magnification ×40 arrows A = large droplet macrosteatosis; arrows B = small droplet macrosteatosis; arrows C = inflammatory cell infiltrate; NM—nutritive milk; Eth—ethanol; ZO—zingerone; W—water; ^{ad}—adult treatment.

B: Females

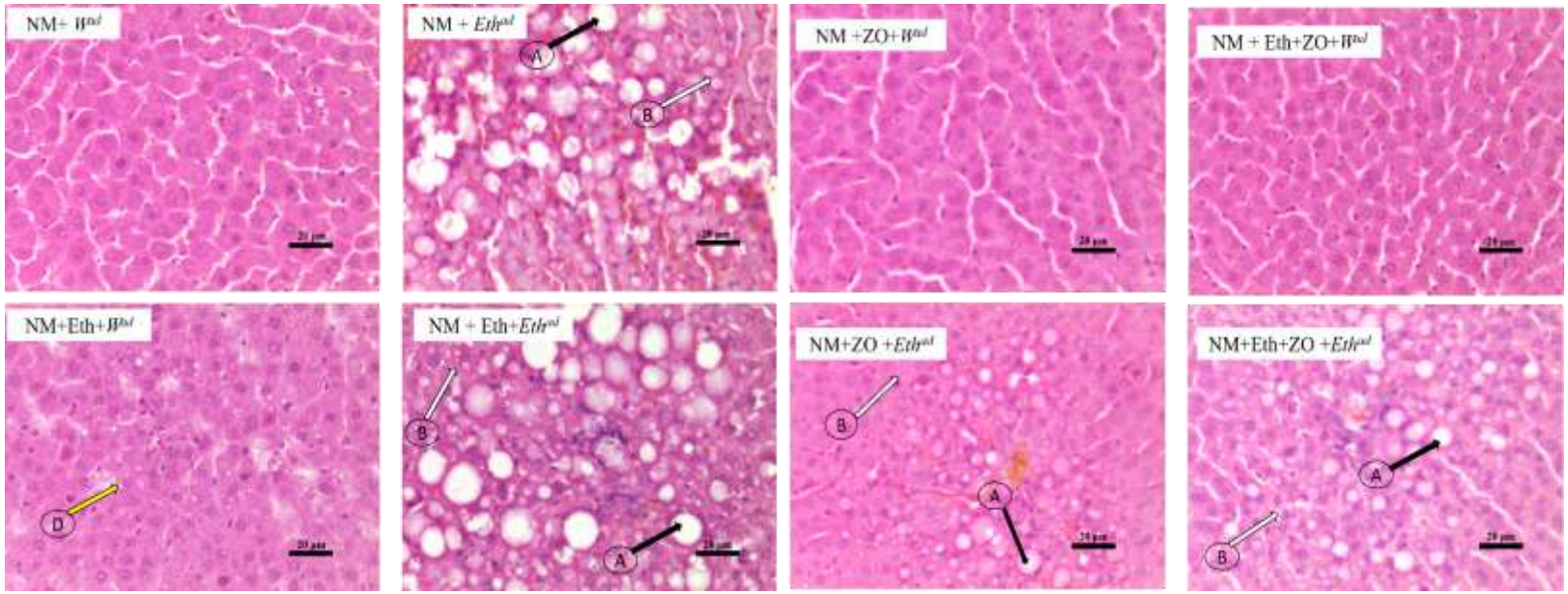

Figure 5.4: Photomicrographs of female (**B**) liver sections stained with H&E. Magnification ×40 arrows A = large droplet macrosteatosis; arrows B = small droplet macrosteatosis; arrows C = inflammatory cell infiltrate; arrow D = microsteatosis. NM—nutritive milk; Eth—ethanol; ZO—zingerone; W—water; ^{ad}—adult treatment.

5.4.5 Effect of neonatal orally administered zingerone on lipid regulatory genes

Treatment regimens had a significant ($p < 0.05$) effect on the mRNA expression level of *PPAR-α* of both male and female rats. Both late single and double hit with alcohol significantly decreased [$p = 0.014$, NM+Eth^{ad} vs NM+W^{ad}; $p = 0.0009$, NM+Eth+Eth^{ad} vs NM+W^{ad} Fig 5.2B] *PPAR-α* expression levels in male rats and they also significantly decreased [$p = 0.040$, NM+Eth^{ad} vs NM+W^{ad}; $p = 0.019$, NM+Eth+Eth^{ad} vs NM+W^{ad} Fig 5.2B] that of females relative to control. Neonatal orally administered zingerone in combination with either a late single or double alcohol hit had no effect on *PPAR-α* expression in male and female rats relative to the control ($p > 0.05$).

Both a late single and double hit with alcohol significantly increased ($p < 0.05$, Fig 5.2C & 5.2F) the *SREBP1c* expression in male and female rats. Neonatal orally administered zingerone in combination with a late single alcohol hit had no effect on *SREBP1c* expression level in male ($p = 0.635$, Fig 5.2C) and female ($p = 0.960$, Fig 5.2F) rats compared to their respective control counterparts. Neonatal orally administered zingerone in combination with a double alcohol hit did had no effect on *SREBP1c* expression level in male rats ($p > 0.05$ vs control) but it significantly increased *SREBP1c* expression in female rats ($p = 0.005$).

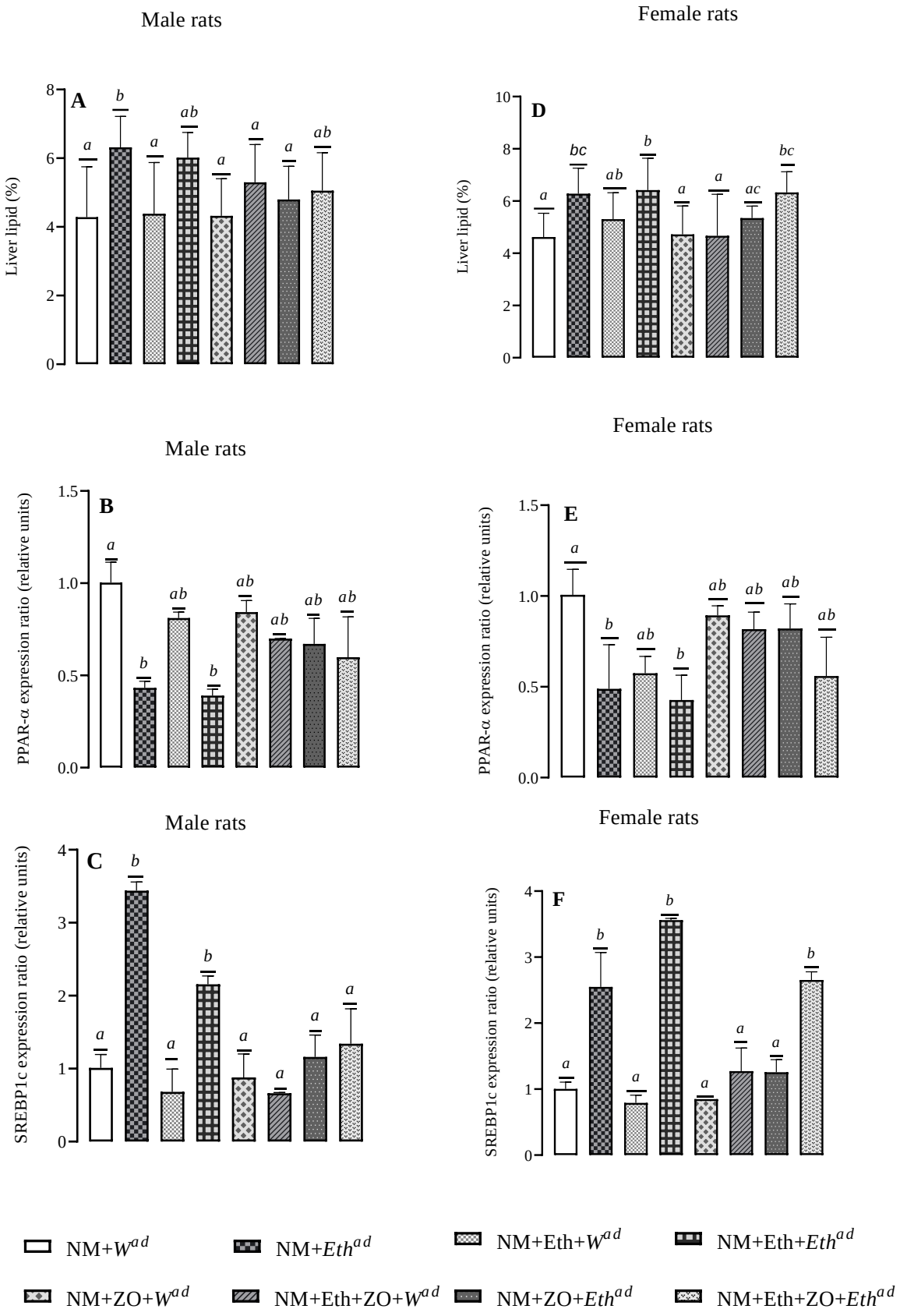
Male rats
Female rats
A
D
Liver lipid (%)
Male rats
Female rats
B
E
PPAR-α expression ratio (relative units)
Male rats
Female rats
C
F
SREBP1c expression ratio (relative units)
NM+W^ad
NM+Eth^ad
NM+Eth+W^ad
NM+Eth+Eth^ad
NM+ZO+W^ad
NM+Eth+ZO+W^ad
NM+ZO+Eth^ad
NM+Eth+ZO+Eth^ad

Figure 5.2: Effect of neonatal orally administered zingerone on liver lipid (A&D); PPAR-α and (B&E) SREBP1c(C&F) gene expression in male and female rats drinking alcohol in adulthood.

Data are presented as Mean ± SD. [ab] = Bars with different letters differ significantly at $p < 0.05$. **Control (NM+W^{ad})** – neonatally gavaged with nutritive milk (NM) and drank plain drinking water (W^{ad}) at stage III; **late single hit (NM+Eth^{ad})** - neonatally gavaged with NM and drank ethanol (Eth^{ad}) solution at stage III; **early single hit (NM+Eth+W^{ad})** - neonatally gavaged with ethanol in nutritive milk and drank plain drinking water (W^{ad}) at stage III; **double hit (NM+Eth+Eth^{ad})** - neonatally gavaged with Eth in nutritive milk and drank Eth^{ad} at stage III; **zingerone alone (NM+ZO+W^{ad})** - neonatally gavaged with ZO in nutritive milk and drank plain drinking water (W^{ad}) at stage III; **early single hit with zingerone (NM+Eth+ZO+W^{ad})** - neonatally gavaged with NM, Eth, ZO and drank plain drinking water (W^{ad}) at stage III; **late single hit with zingerone (NM+ZO+Eth^{ad})** - neonatally gavaged with NM and ZO and drank Eth^{ad} at stage III; **double hit with zingerone (NM+Eth+ZO+Eth^{ad})** - neonatally gavaged with NM, Eth and ZO and drank Eth^{ad} at stage III;[ad] - adult treatment; NM-Nutritive milk; Eth-ethanol; ZO-zingerone; W-water; n = 6-8 per treatment group for liver lipids. For lipid regulatory genes, *PPAR-α and SREBP1c*; n=2-3 rats per treatment group.

5.4.6 Effect of neonatal orally administered zingerone on plasma liver enzyme activities

In male and female rats, treatment regimen had no significant effect ($p > 0.05$ vs control) on plasma AST and ALT activities (Table 5.2).

Table 5.2:**Effect of neonatal orally administered zingerone on plasma liver enzyme activities in adult male and female rats drinking alcohol in adulthood**

	Males		Females	
Treatment Group	**AST(U/L)**	**ALT (U/L)**	**AST (U/L)**	**ALT (U/L)**
NM+W^{ad}	247.8±155.5[a]	61.8±11.3[a]	147.2±35.9[a]	57.0±19.5[a]
NM+Eth^{ad}	298.8±130.4[a]	114.0±47.2[a]	168.2±46.6[a]	62.8±22.2[a]
NM+Eth+W^{ad}	210.6±81.8[a]	96.2±36.2[a]	161.3±77.0[a]	50.8±19.5[a]
NM+Eth+Eth^{ad}	156.4±46.2[a]	81.0±30.3[a]	200.7±74.6[a]	69.5±32.6[a]
NM+ZO+W^{ad}	183.3±45.7[a]	70.3±21.2[a]	145.0±58.3[a]	51.8±18.9[a]
NM+Eth+ZO+W^{ad}	224.6±115.3[a]	79.8±28.1[a]	121.4±28.3[a]	43.0±7.3[a]
NM+ZO+Eth^{ad}	207.1±102.6[a]	75.8±25.8[a]	117.6±14.1[a]	46.8±9.4[a]
NM+Eth+ZO+Eth^{ad}	228.7±75.81[a]	95.8±28.7[a]	165.0±69.5[a]	46.83±7.5[a]

Data is presented as Mean ±SD. [aa] = Within column means that do not differ significantly at p> 0.050. **Control (NM+W^{ad})** – neonatally gavaged with nutritive milk (NM) and drank plain drinking water (W^{ad}) at stage III; **late single hit (NM+Eth^{ad}) -** neonatally gavaged with NM and drank ethanol (Eth^{ad}) solution at stage III; **early single hit (NM+Eth+W^{ad}) -** neonatally gavaged with ethanol in nutritive milk and drank plain drinking water (W^{ad}) at stage III; **double hit (NM+Eth+Eth^{ad}) -** neonatally gavaged with Eth in nutritive milk and drank Eth^{ad} at stage III; **zingerone alone (NM+ZO+W^{ad}) -** neonatally gavaged with ZO in nutritive milk and drank plain

drinking water (W^{ad}) at stage III; **early single hit with zingerone (NM+Eth+ZO+W^{ad})** - neonatally gavaged with NM, Eth, ZO and drank plain drinking water (W^{ad}) at stage III; **late single hit with zingerone (NM+ZO+Eth^{ad})** - neonatally gavaged with NM and ZO and drank Eth^{ad} at stage III; **double hit with zingerone (NM+Eth+ZO+Eth^{ad})** - neonatally gavaged with NM, Eth and ZO and drank Eth^{ad} at stage III;^{ad}-adult treatment ; NM-Nutritive milk; Eth-ethanol; ZO-zingerone; W-water; n = 5-6 per treatment group.

5.4.7 Effect of neonatal orally administered zingerone on plasma CYP2E1 and hepatic TBARS

Ethanol consumption increased ($p < 0.05$, Fig 5.5A & 5.5C) CYP2E1 concentration in male and female rats that had late single hit and double hit with ethanol. Neonatal orally administered zingerone in combination with either late single hit or double hit with alcohol significantly increased CYP2E1 concentration of male and female rats relative to control. Treatment regimens had no effect on hepatic TBARS concentration in male ($p = 0.096$, Fig 5.5B) and female rats ($p = 0.050$, 5.5D).

5.4.8 Effect of neonatal orally administered zingerone on biomarkers of inflammation

Treatment regimens had no effect ($p > 0.05$ vs control) on plasma TNF-α and IL-6 concentrations and hepatic mRNA expression of *NFK-β* and *TNF-α* male rats and female rats (Fig 5.6 A-H).

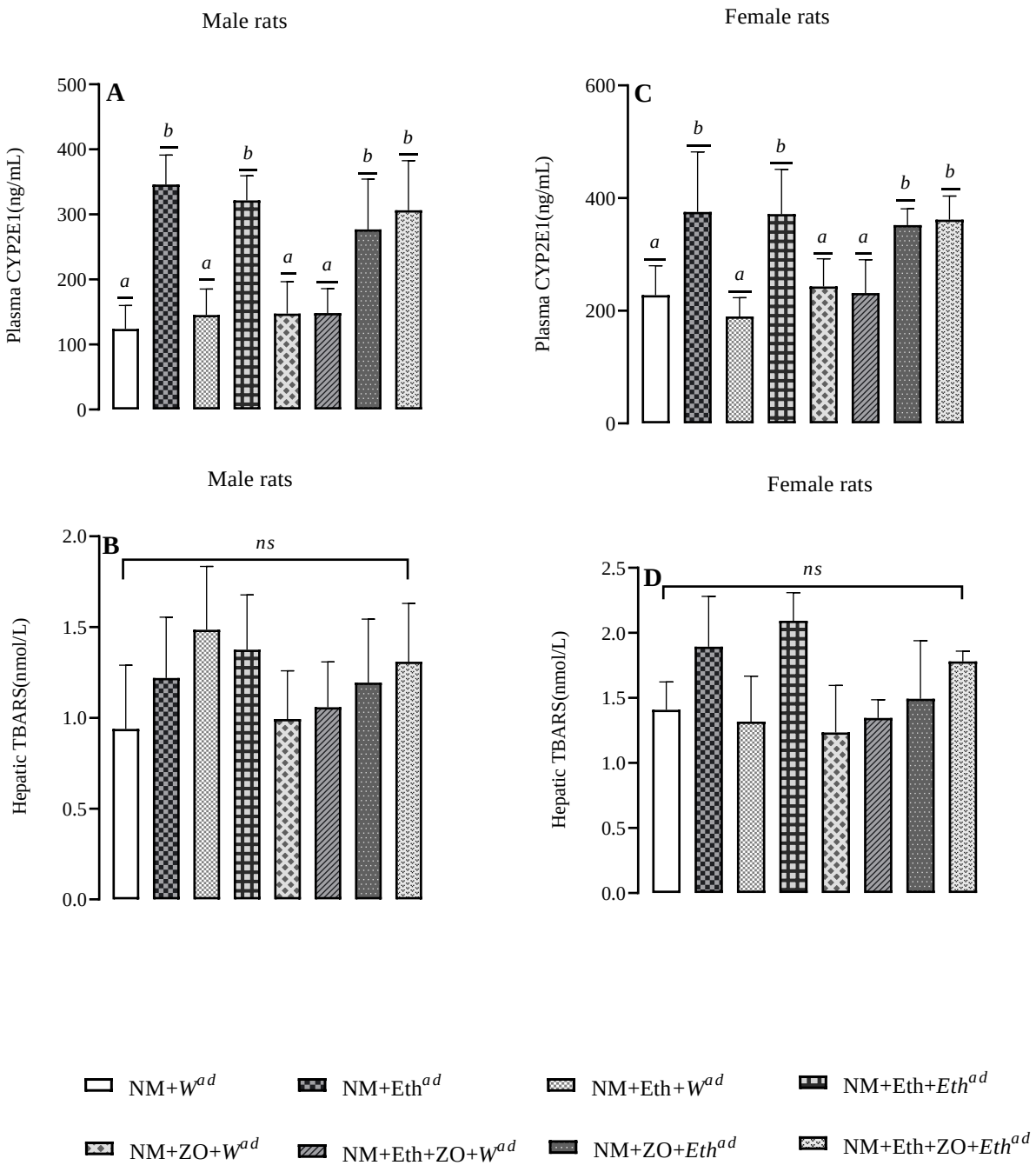
Male rats
Female rats
A
C
B
D
Plasma CYP2E1(ng/mL)
Hepatic TBARS(nmol/L)
ns
NM+W^ad
NM+Eth^ad
NM+Eth+W^ad
NM+Eth+Eth^ad
NM+ZO+W^ad
NM+Eth+ZO+W^ad
NM+ZO+Eth^ad
NM+Eth+ZO+Eth^ad

Figure 5.5: Effect of neonatal orally administered zingerone on plasma CYP2E1 (A&C) concentration and hepatic TBARS (B&D) concentrations in male (A&B) and female (D&D) rats drinking alcohol in adulthood.

Data are presented as Mean ± SD. ($p<0.05$). [ab] = Bars means with different letters are significantly different at $p < 0.05$ **Control (NM+W^{ad})** – neonatally gavaged with nutritive milk (NM) and received plain drinking water (W^{ad}) at stage III; **late single hit (NM+Eth^{ad})** - neonatally gavaged with NM and received ethanol (Eth^{ad}) solution at stage III; **early single hit (NM+Eth+W^{ad})** - neonatally gavaged with ethanol in nutritive milk and received plain drinking water (W^{ad}) at stage III; **double hit (NM+Eth+Eth^{ad})** - neonatally gavaged with Eth in nutritive milk and received Eth^{ad} at stage III; **zingerone alone (NM+ZO+W^{ad})** - neonatally gavaged with ZO in nutritive milk and received plain drinking water (W^{ad}) at stage III; **early single hit with zingerone (NM+Eth+ZO+W^{ad})** - neonatally gavaged with NM, Eth, ZO and received plain drinking water (W^{ad}) at stage III; **late single hit with zingerone (NM+ZO+Eth^{ad})** - neonatally gavaged with NM and ZO and received Eth^{ad} at stage III; **double hit with zingerone (NM+Eth+ZO+Eth^{ad})** - neonatally gavaged with NM, Eth and ZO and received Eth^{ad} at stage III; [ad]-adult treatment; NM-Nutritive milk; Eth-ethanol; ZO-zingerone; W-water; n = 6-8 per treatment group for plasma TNF-α and IL-6. *TNF-α and NF-KB*; n=2-3 rats per treatment group

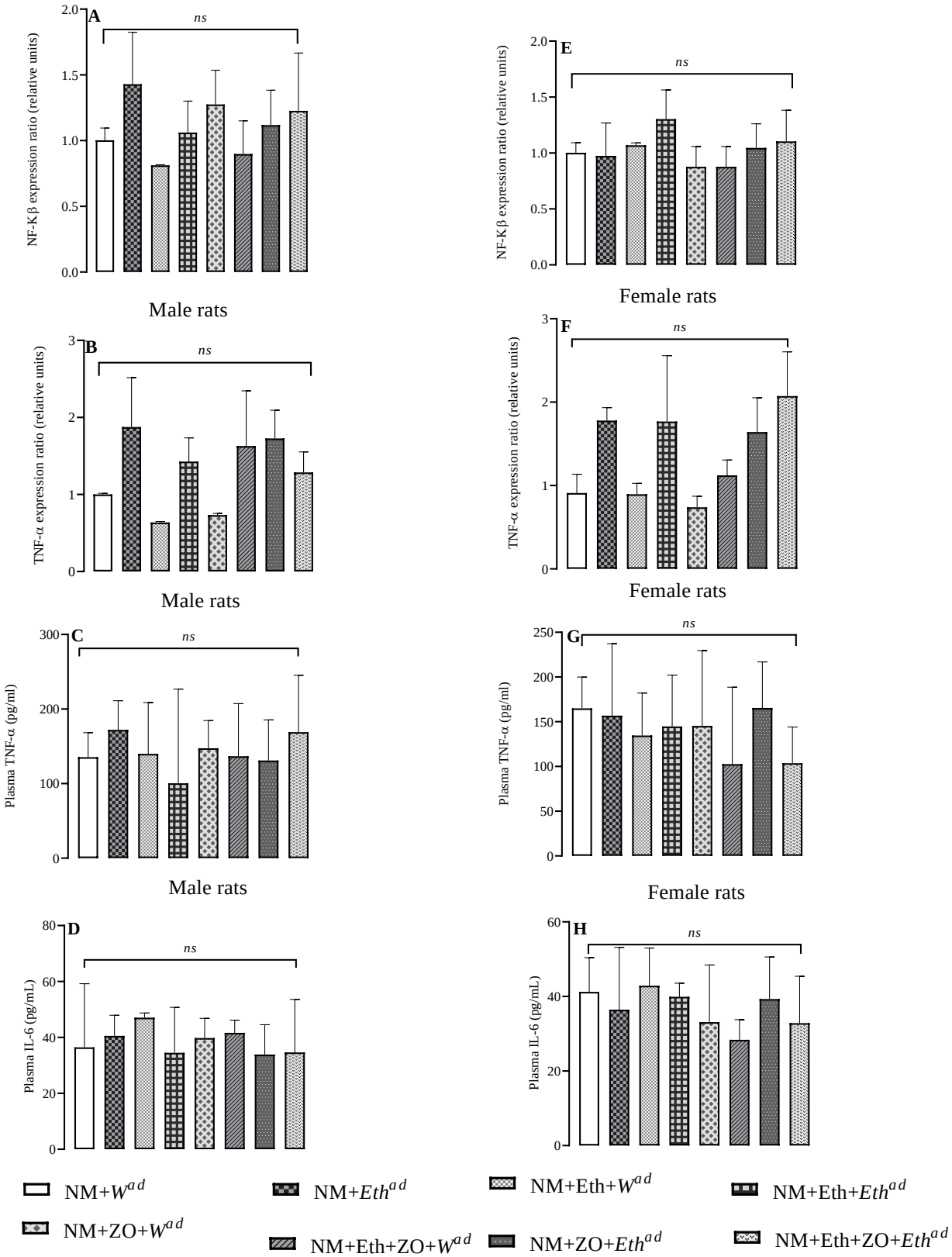
Male rats
Female rats
A
E
B
F
C
G
D
H
ns
NF-Kβ expression ratio (relative units)
TNF-α expression ratio (relative units)
Plasma TNF-α (pg/ml)
Plasma IL-6 (pg/mL)
NM+W^ad
NM+Eth^ad
NM+Eth+W^ad
NM+Eth+Eth^ad
NM+ZO+W^ad
NM+Eth+ZO+W^ad
NM+ZO+Eth^ad
NM+Eth+ZO+Eth^ad

Figure 5.6: **Effect of neonatal orally administered zingerone on *NF-Kβ* gene expression (A&E), *TNF –α* (B&F) gene expression, plasma *TNF-α* (C&D) and plasma IL-6 (D&H) concentrations in male and female rats drinking alcohol in adulthood**.

Data are presented as Mean ± SD. [ab] = Bars with different letters are significantly different at p< 0.05. **Control (NM+W^{ad})** – neonatally gavaged with nutritive milk (NM) and drank plain drinking water (W^{ad}) at stage III; **late single hit (NM+Eth^{ad})** - neonatally gavaged with NM and drank ethanol (Eth^{ad}) solution at stage III; **early single hit (NM+Eth+W^{ad})** - neonatally gavaged with ethanol in nutritive milk and drank plain drinking water (W^{ad}) at stage III; **double hit (NM+Eth+Eth^{ad})** - neonatally gavaged with Eth in nutritive milk and drank Eth^{ad} at stage III; **zingerone alone (NM+ZO+W^{ad})** - neonatally gavaged with ZO in nutritive milk and drank plain drinking water (W^{ad}) at stage III; **early single hit with zingerone (NM+Eth+ZO+W^{ad})** - neonatally gavaged with NM, Eth, ZO and drank plain drinking water (W^{ad}) at stage III; **late single hit with zingerone (NM+ZO+Eth^{ad})** - neonatally gavaged with NM and ZO and drank Eth^{ad} at stage III; **double hit with zingerone (NM+Eth+ZO+Eth^{ad})** - neonatally gavaged with NM, Eth and ZO and drank Eth^{ad} at stage III; [ad]-adult treatment;NM-Nutritive milk; Eth-ethanol; ZO-zingerone; W-water; n = 6-8 per treatment group for plasma TNF-α and IL-6. Gene expression for *TNF-α and NF-Kβ*; n=2-3 rats per treatment group

5.5 Discussion

In this study, we investigated the effect of neonatal orally administered zingerone on early and late single and a double hit with alcohol on the development of alcohol-induced fatty liver disease in adulthood. A late single hit and double hit with alcohol in male and female rats resulted in an increased liver fat content accompanied by macrosteatosis, downregulation of *PPAR-α* and

upregulation of *SREBP1c*. Neonatal orally administered zingerone attenuated fat accretion by preventing an upregulation in *SREBP1c* in male rats. It also mitigated hepatic steatosis and the downregulation of *PPAR-α* induced by late single or double alcohol hit in male and female rats. Due to the small sample size for the molecular analysis, caution must be exercised in the fine interpretation of the data.

Our findings showed that neonatal administered ethanol and zingerone had no effect on ethanol consumption of the rats in adulthood. Previous studies reported that exposure to alcohol during the prenatal developmental phase did not affect adolescent ethanol consumption because of aversion to alcohol developed as a result of prenatal exposure (Hilakivi, 2009; Biggio et al., 2018). However, other research revealed that prenatal exposure to ethanol via food and substrate exchange between maternal and foetal blood in the placenta increases the likelihood of increasing alcohol intake in adolescence and adulthood because it enhances the brain's reward system (Bordner & Deak, 2015; Nizhnikov et al., 2016). Findings from the current study suggest that the aversive as well as the appetitive chemo-sensory stimuli to alcohol were not modified by neonatal orally administered ethanol and/or zingerone. However, we did not assess the neurochemical changes induced by the neonatal exposure to the interventions, hence there is need for further investigations into the neurochemical changes induced by exposure to alcohol and zingerone during the neonatal growth phase.

Early, late single hit and double hit with alcohol had no effect on the hepatosomatic index in the present study. In line with our finding, another research group found that alcohol consumption does not affect the hepatosomatic index of rats (van de Wiel et al., 1990). However, in contrast to our findings, other studies showed that alcohol consumption increased liver weight (Al-Humadi et

al., 2019; Rasineni et al., 2019). The studies of AL-Humadi et al. (2019) and Rasineni et al. (2019) made use of the Lieber-DeCarli alcohol liquid diet, which contains 35.5% compared to Labchef standard rat chow (Epol®, Johannesburg, South Africa) with 5% fats with alcohol solution as a drinking fluid used in this study.

A late single hit (both sexes) and double hit (females only) with alcohol increased the liver fat content. Alcohol disrupts several aspects of hepatic lipid flux that leads to lipid accumulation, including activation of *SREBP1c* to stimulate lipogenesis (You & Arteel, 2019). Studies report that alcohol causes hepatic lipid accumulation which may result in the development of steatosis (You et al., 2002; Ji et al., 2008). Hence, it was not surprising that alcohol-induced increase in liver fat content was accompanied with the formation of prominent small and large droplet macrosteatosis in both male and female rats. This observation was associated with peroxisome proliferator activator receptor-alpha (*PPAR-α*) downregulation and sterol regulatory element binding protein 1c (*SREBP1*c) upregulation. Previous studies reported that alcohol consumption caused alcohol-induced fatty liver by downregulating *PPAR-α* (Park et al., 2014). Furthermore, ethanol decreases the AMP-activated protein kinase (AMPK)–Sirtuin (SIRT) signaling pathway and its downstream signalling proteins resulting in the upregulation of *SREBP1c* (Jiang et al., 2015). These proteins, *PPAR-α* and *SREBP1c*, are involved in regulating the hepatic fatty oxidation pathway and lipid droplet formation (Gu et al., 2015; Straub, 2015). Therefore, it is also plausible that in the present study, hepatic macrosteatosis was caused by downregulation and upregulation in *PPAR-α* and *SREBP1c*, respectively.

Neonatal orally administered zingerone mitigated the alcohol-induced downregulation of hepatic *PPAR-α* expression in male and female rats but protected against *SREBP1c* upregulation in male rats only and attenuated the accretion of liver fat and the formation of large droplet macrosteatosis,

especially in the male rats. Currently, we do not have an explanation for the sexual dimorphic effect of zingerone on *SREBP1c* expression, thus further investigation is required. Nonetheless, preceding studies have reported that sexual dimorphism in rats can be attributed to variance in rate and pattern of early development and responses to insult in male and female rats (Sundrani et al., 2017; Lembede et al., 2018). *In vivo* zingerone activates *PPAR-α* (Chung et al., 2009). It is therefore possible that it exerted its anti-steatotic effects by preventing the downregulation of *PPAR-α* Additionally, previous research had found that zingerone can supress the expression of *SREBP1c* (Mohammed, 2022) to protect against fatty liver formation and lipid accumulation therefore our findings are in tandem with other research outcomes.

Steatosis is generally reversible after ethanol withdrawal (Seitz et al., 2018). However, we observed microsteatosis in the livers of female rats that had an early single hit with alcohol which was neither accompanied with a significant increase in liver fat content nor an effect on the lipid regulatory genes. In line with our findings, Shen et al. (2014) also reported that adult female rat offspring prenatally exposed to ethanol also exhibited microsteatosis. Presently, it is difficult to provide an explanation to this phenomenon. In our recent study we observed that oral administration of ethanol during the suckling period reduced the plasma triglyceride concentration of female rat pups (Asiedu et al., 2022). We therefore speculate that the observed microsteatosis is likely due to the neonatal alcohol-induced export of triglycerides from the plasma to the liver.

In the current study, hepatic lobular inflammation and necrosis were not evident in the liver sections of male and female rats suggesting that none of the interventions caused liver inflammation. In agreement with the histological data, inflammatory biochemical markers (TNF-α, IL-6 and NF-Kβ) showed no significant results. Our finding is consistent with other studies that showed that in rodents the consumption of alcohol only did not induce significant hepatic

inflammation and necrosis (Larosche et al., 2009), except under prolonged (29 weeks) consumption of 40% ethanol (Keegan et al., 1995). Additionally, the rats only developed simple steatosis without a progression to alcoholic steatohepatitis. Simple steatosis is not associated with inflammation (Greuter et al., 2017; Mazzolin et al., 2020; Heeren et al., 2021), though given more time this could have progressed to steatohepatitis. Plasma AST and ALT activities are considered surrogate biomarkers for liver injury (Morales-González et al., 2018). Ethanol consumption that caused hepatocyte inflammation and necrosis was shown to be associated with increased plasma AST and ALT activity (Huang et al. 2010) and Yamasaki et al. (2019) reported no change in AST and ALT activity without inflammation and necrosis. However, Radic et al. (2019) observed that despite causing hepatic focal inflammation and mild necrosis, the consumption of ethanol did affect on plasma AST and ALT activity of rats suggesting poor correlation between liver enzyme activity and damage (Morales-Gonzalez et al., 1999). Our findings showed similarities in histological findings on hepatocytes with no evidence of necrosis and inflammation which explains similarities in plasma ALT and AST concentration of the rats since there was no liver cell damage. The present data indicate that eight weeks of ethanol consumption by the rats was not sufficient to induce liver cell injury. Importantly, we show that neonatal orally administered zingerone did not cause liver inflammation and injury and this findings is in agreement with its previously reported effects in adult rats (Cheong et al., 2016; Narayanan & Jesudoss, 2016; Mani et al., 2016).

Our findings showed that ethanol consumption in the late single and double hit groups elevated CYP2E1 concentration of the rats. CYP2E1 catalyses the metabolism of alcohol (Osna et al., 2017) in the liver. Chronic ethanol consumption has been shown to induce CYP2E1 activity in order for it to facilitate ethanol metabolism (Osna et al., 2017). Additionally, ethanol has been reported to

induce and stabilize CYP2E1 proteins post-translationally (Roberts et al., 1995). Contrary to our finding, Kolata et al. (2019) did not observe an increased CYP2E1 protein in male Wistar rats that consumed 10% ethanol solution for 6 weeks. This variance might be because we used 20% ethanol; a concentration which was 100% higher. Furthermore, the current study used Sprague-Dawley rats which could have also brought differences in rat strain responses. In the current study, we observed that neonatal orally administered zingerone did not affect the alcohol-induced increase in CYP2E1 proteins. Acute pharmacokinetic study of zingerone in rats via mass spectrophotometry demonstrated that it does not significantly affect of the microsomal content of P450 enzymes (Huang, 2010). Therefore, zingerone cannot be used to promote alcohol abstinence as the induction of CYP2E1 is associated with greater tolerance to high ethanol intake (Osna et al., 2017).

The induction of CYP2E1 is associated with alcohol-induced oxidative stress via the generation of acetaldehyde and increased production of free radical species, which can form adducts with DNA and cause oxidative tissue injury (Osna et al., 2017). However, our findings recorded similar hepatic TBARS concentration across treatment regimens which suggests no initiation of significant lipid peroxidation in both male and female rats. Our findings are in agreement with the works of Kołota et al. (2020) and Radic et al. (2019) but at variance with Teare et al. (1994) and Keegan et al. (1995) possibly due to the difference in experimental duration and the development of more advanced stages of ALD. Therefore, our findings corroborate the hypothesis that oxidants from CYP2E1 play a minor role in the mechanisms involved in the early stages of ALD (Kono et al., 1999). Although an extended alcohol consumption or a more sensitive biomarker like F2-isoprostanes might have yielded result as TBARs are not considered sensitive and reliable biomarkers of lipid peroxidation due to their reactivity and metabolism (Milne, 2017). Late single and double hit with alcohol increased hepatic TBARs by 44.1% and 52.0%, respectively in the

female rats. However, neonatal orally administered zingerone reduced TBARS by 27.1% and 23.7% in the late single and double alcohol hit groups, respectively. Our study corroborate previous reports that indicate that zingerone markedly reduces lipid peroxidation via its free radical scavenging ability (Vinothkumar et al., 2014; Mani et al., 2016). While it has been previously reported that zingerone reduces high-fat diet induced steatosis and its associated inflammation, what is not known is its ability to programme for long-term protection against alcohol-induced fatty liver disease.

5.6 Conclusion

This present study showed that late and double hit with alcohol in rats resulted in the development of AFLD characterised by small and large droplet macrosteatosis, with a downregulation in *PPAR-α* and an upregulation in *SREBP1c* without significant inflammatory changes or elevation in liver enzymes. Alcohol fatty liver disease renders the liver susceptible to toxic effects of alcohol and other insults However, neonatal oral administration of zingerone attenuated AFLD in a sex-dependent manner. Zingerone can therefore be strategically administered in the neonatal phase as a potential prophylactic agent for its beneficial effects against AFLD and ability to blunt the development of ALD; and lessen the burden of ALD on the healthcare system. This information should be taken into consideration when developing guidelines regarding alcohol consumption during breastfeeding. Additionally, this study recommends fortifying diets of breastfeeding mothers with ginger, a rich source of zingerone for its prophylactic benefits against diseases.

The next chapter gives a description, in a summarised and consolidated manner, the information given in the preceding three experimental manuscript based chapters. The chapter gives a general

consolidated discussion and highligths the key conclusions drawn from the study, points to study limitations and recommends areas which further studies can focus on.

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CHAPTER SIX- GENERAL DISCUSSION, CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

This study sought to determine the metabolic phenotype and oxidative status of rat pups exposed to ethanol during the postnatal stage and whether neonatal oral co-administration of zingerone can mitigate the effect of exposure to alcohol during the neonatal growth phase. Furthermore, the study sought to evaluate whether neonatal orally administered zingerone programmed for protection against early, late and double hits with alcohol in adulthood.

6.1 Acute study: findings, discussions and conclusions

The major findings of the rat pup study (experiment 1) showed that alcohol, orally administered during the neonatal growth phase, significantly elevated hepatic CYP2E1 and decreased total hepatic glutathione in male rat pups but significantly reduced plasma triglyceride concentration in female rat pups. Low plasma triglycerides in the female rat pups was initially ignored due to low-fat adiposity in rat pups (Tavares do Carmo et al. 1996). However, it is possible that the plasma triglycerides were being transported into the liver, but liver histology of the rat pups was not determined in the short-term study. In the second experiment, female rats subjected to an early hit with ethanol during their neonatal growth phase developed microsteatosis. Alochol-induced fatty liver disease is generally reversible with alcohol withdrawal after two weeks in human and murine studies (Seitz et al. 2018; Thomes et al. 2019). Interestingly, their male rat pup counterparts that had a decrease in hepatic glutathione did not exhibit microsteatosis in adulthood. Apparently, glutathione depletion confers protection against alcohol-induced fatty liver disease via oxidative stress adaptive response that activate the AMPK pathway (Chen et al., 2016).

Neonatal orally administered zingerone by itself had similar effects as the control group but when co-administered orally, with ethanol it did not protect against alcohol-induced hepatic increase in

CYP2E1 and decrease in GSH concentrations in the male rat pups. This outcome wherein zingerone fails to mitigate oxidative stress is not unique to this study. In their study using mice models, Kabuto and Yamanushi (2011) observed that when co-administered with 6-hydroxydopamine zingerone failed to prevent oxidative-inflammatory disease. This suggests that at disease onset, the rate at which antioxidants are produced and the free radical scavenging activity of the administered zingerone is low when co-administered simultaneously with an insult. Regarding findings from the current study, it is essential to note that compared to zingerone, a relatively less soluble substance, the absorption of alcohol, a hydrophilic substance, is less impeded in the gut (Takizawa et al., 2012). Thus, alcohol gets absorbed and transported into hepatocytes faster than zingerone.

Pharmacokinetic studies on zingerone absorption, distribution and elimination after oral administration are limited. Huang (2010) showed that oral administration of 32 mg/kg body weight of zingerone gets rapidly absorbed into the blood circulation with bioavailability of 72.2% and plasma protein binding of 52% in rats. The absorbed zingerone then distributes into several organ systems, including the liver, pancreas and kidney (Huang, 2010; Li et al., 2019). Due to its lipophilic nature and the possibility of having to go through several bypasses prior to its metabolism in the liver, zingerone notably takes a longer time to reach the tissue (Huang, 2010; Li et al., 2019). The reported failure by zingerone to protect against alcohol-induced hepatic increase in CYP2E1 and decrease in GSH in the current study could be due to its delayed entry into the hepatic cells in comparison to alcohol which quickly diffuses into body compartments, especially the highly perfused liver.

6.2 Chronic study: findings, discussions and conclusions adult intervention: Alcohol-induced metabolic and fatty liver changes

In vivo the rate at which zingerone is eliminated from tissues is slow (Li et al. 2019). Only 51% of zingerone is excreted through bile acids, faeces and urine within 24 hours (Huang, 2010). This observed delay in elimination warrants the use of zingerone as a pre-treatment agent (Alibakhshi et al. 2018; Safhi 2018; Soliman, Anees, and Ibrahim 2018). One study demonstrated that pre-treatment with zingerone is more effective than co-treatment (Kabuto, Tada, and Kohno 2007) but Mir et al. (2018) claim that there is no difference whether it is used as pre-treatment or co-treatment drug. However, a careful observation showed that pre-treatment with zingerone produced higher antioxidants and better response to cyclophosphamide-induced hepatoxicity (Mir et al., 2018). Therefore, in the current study it is likely the use of zingerone as a prophylactic agent resulted in epigenetic changes that enhanced its therapeutic potential than when used as a co-treatment or post-treatment drug against disease. Evidently, it can be inferred from the current study that intervening with zingerone during the neonatal growth phase and then allowing the rats to grow into adolescence/adulthood might have mediated zingerone-induced epigenetic remodelling, hence offering protection against the development of liver lipid accumulation and hepatic steatosis via modulation of hepatic lipid genes; *PPAR-α* and *SREBP1c*.

In the second phase of the experiment, when alcohol was consumed by half of the rats, most metabolic responses were not abnormally altered. Ethanol consumption did not affect gain in body mass, linear growth by measurement of the tibia length, insulin, leptin, adiponectin concentrations, and visceral adiposity in both male and female rats. The relationship between cardio-metabolic risk factors and ethanol consumption is J-shaped, where light-moderate ethanol consumption improves while a heavy ethanol consumption increases the risk of cardiovascular disease (Joosten et al. 2011; Shimomura and Wakabayashi 2013; Metcalf, Scragg, and Jackson 2014). Although a high (>5 g/kg/bwt) ethanol consumption (Leeman et al. 2010) was achieved by the method

employed in this study, this high level was continuous and not in bouts, hence possibly decreasing the blood alcohol concentration. Drinking a large volume of alcohol at a time increases contact area with stomach, thus increasing blood alcohol concentration momentarily (Borowitz et al. 1971), although recent publication indicate that the concentration of alcohol is a better indicator of blood alcohol concentration than the volume of alcohol consumed (Dilley et al. 2018). There was therefore no significant impact on most of the cardio-metabolic parameters measured. Additionally, there was no liver injury, hepatic inflammation or lipid peroxidation in male and female rats that consumed alcohol during the second intervention stage. Firstly, the induction of CYP2E1 might have facilitated fast catabolism of ethanol, alleviating the impact of ethanol consumed by the rats. Consistently, studies that used the ethanol *ad libitum* method demonstrated no considerable effects on cardio-metabolic disorders (Castro et al. 2013; Justice et al. 2019; Radic et al. 2018; Yamasaki et al. 2019). Secondly, difference in rat strain could have been a contributory factor. Variations in ethanol metabolizing enzymes (ADH and CYP2E1) have been reported to contribute to inter-strain differences in the efficiency of ethanol detoxification (DeNucci et al. 2010). Additionally, the use of young rats (about 4 months old at study termination) in the current study might have contributed to less damage from ethanol consumption since it has been demonstrated that young rats are resistant to alcohol-induced liver oxidative injury and metabolic alteration because of higher regenerative potential in the young compared to the aged rats (Biondo-Simões et al. 2006; Schmucker and Sanchez 2011; Xiao et al. 2019; Smith et al. 2022). With ageing, the hepatic activity of CYP2E1 and ADH decreases, water distribution volume decreases and gastric atrophy occurs leading to high blood ethanol concentration and hence more damage (Meier and Seitz 2008). With the ethanol *ad libitum* feeding model in Wistar rats, studies that reported alcoholic steatohepatitis(ASH) with fibrosis and significant inflammation either used a

long ethanol feeding protocol of 29 weeks (Keegan et al., 1995) or 25 weeks (Best and Hartroft 1949) or aged 60 weeks old rats for 8 weeks (Brandon-Warner et al. 2012). Hence the rats were sacrificed at least after 6 months compared to those used in this study. Age indeed does have a significant role in the development and progression of ALD. However, the current study used young adult rats because the target was to assess the effect of ethanol intake exclusively without having age as a confounding factor especially considering the fact that alcohol consumption starts at a young age in humans (Spear 2015).

Human and murine studies have showed that development of fatty liver disease occur in young adults without liver injury and inflammation and are mostly asymptomatic (Fontana et al., 2013; Flemming et al., 2021). The progression of AFLD to ASH occurs gradually over years without clinical symptoms. Accordingly, data on liver health from the current study showed that a late single hit and double hit with alcohol resulted in significant liver lipid accretion accompanied with large droplet macrosteatosis without hepatocyte inflammation or damage but with significant decrease in *PPAR-α* and increase in *SREBP1c* expressions. This typified the early-stage of alcohol-induced liver disease; alcoholic fatty liver disease. Neonatal orally administered zingerone resulted in a significant reduction in visceral fat mass in female rats. Zingerone stays longer in fat cells than in other tissues (Huang, 2010) hence its effect on lipids is much more effective. In agreement with the impact of zingerone on visceral fat accumulation, hepatic lipid accretion and steatosis were evidently mitigated in both male and female rats with concurrent modulation of the lipid regulatory genes; *PPAR-α* and *SREBP1c*.

6.3 Conclusions

In summary, this study showed that neonatal oral alcohol administration only did not impact ethanol consumption volumes, feed intake or modulate any lipid regulatory genes in male or female rats, but an early single hit in females resulted in microsteatosis. A late single and double hit with ethanol resulted in a significant increase in liver lipid content and dysregulated lipid regulatory genes (*PPAR-α and SREBP1c*) leading to the development of large droplet macrosteatosis in both sexes. Additionally, a late single hit with ethanol significantly reduced feed, fluid and ethanol intake with concomitant elevation in plasma CYP2E1. All of these changes occurred without overt metabolic disturbances. Meanwhile, neonatal orally administered zingerone attenuated the formation of large droplet macrosteatosis in male and female rats. Zingerone also reduced liver lipid content via upregulation *PPAR-α* and downregulation of *SREBP1c* without affecting alcohol-induced reduction in feed and fluid intake or increase in CYP2E1 proteins.

Although extrapolation of animal studies may be tenuous, this study recommends the use of zingerone in the neonatal period as a prophylactic agent against the consequential development of alcohol-induced fatty liver disease.

6.4 Limitations and future directions

Human neonates get exposed to alcohol through breast milk. However, offspring metabolic outcomes and growth may be affected by the impact of ethanol administration to the lactating dam due to alteration in mother-infant interaction and decreased production of milk (Pepino et al., 2007), a situation that mimics what happens in alcohol-drinking nursing mothers (Costa et al.

2014). Hence, in the current study alcohol solution was not offered to the rat dam to delineate the impact of ethanol on the dam from the offspring.

To induce alcohol-induce liver disease in adulthood, half of the rats were placed on ethanol solution and the other half on plain drinking water as the sole drinking fluid for 8 weeks, a model adopted Ojeda et al. (2008). Adult rats have a natural aversion to alcohol (Lamas-Paz et al. 2018) hence lower concentrations of 5% and 10% were offered to the rats to get them used to the taste of alcohol. Indeed, moving to 20%, ethanol solution did not make any difference to the intake volume and eventually resulted in a high ethanol intake of above 5 g/kg/day (Leeman et al. 2010). This model was suitable given the large number of rats used in this study and typifies the drinking pattern of humans (Lamas-Paz et al. 2018), although, it has the limitation of not achieving high blood alcohol levels and may likely induce malnutrition (Lamas-Paz et al. 2018). Ethanol consumption resulted in a significant decrease in feed intake, but growth performance was not affected, signifying that energy and protein intake were adequate to meet the requirements for normal growth. A diet with 10% protein is recommended for normal rat growth (National Research Council (US) Subcommittee on Laboratory Animal Nutrition 1995). The standard rat chow used in this study contained 20% protein therefore the growth of the rats was not affected.

Other studies used the Lieber-DeCarli diet for ALD studies in rats. However, this diet has shortfalls, which we found unsuitable as the insult of interest was alcohol. Fat constitute 36% of the Lieber-DeCarli alcohol diet (Guo et al. 2018). When alcohol is given as a liquid diet, most of the fats that accumulate in the liver are actually dietary fat (Guo et al. 2018). To circumvent this effect, a low-fat diet of 5 or 12% monounsaturated fat may be used (Guo et al. 2018) as unsaturated fat diet is required to develop ALD (Guo et al. 2018). In the current study a conscious decision was made to choose the use of the ethanol *ad libitum* feeding model with normal rat chow

containing 5% dietary fat in order to eliminate the confounding effect associated with the Lieber-DeCarli alcohol diet. Ethanol solution results in the production of extra calories hence some studies used an isocaloric diet in the control animals (Probyn et al. 2013; Gårdebjer et al. 2015, 2018; Amos-Kroohs et al. 2018) to compensate for the extra calories from alcohol. However a comparison between ethanol pair-fed controls and normal rat chow controls showed no significant difference (Tong et al. 2014) therefore, there was no need to use isocaloric control animals.

A significant limitation of this study was the failure to measure the blood ethanol levels. However, blood alcohol concentration varies widely based on timing of measurement after administration, hence ethanol metabolite such as ethyl glucuronide could be assessed in future studies. Secondly, regarding histological assessment, lipid droplet formation proteins like perilipin 2 and 3 could be useful in the analysis of steatosis. Additionally, failure to quantify hepatic triglycerides in the adult rats was a limitation. Given the borderline results of some analytes (e.g. TBARS & mRNA expression of *TNF-α* in females), an extension of the adult intervention phase could have yielded significant results, thus future studies could consider a longer experimental period. Additionally, more sensitive biomarkers, for example F2-isoprostanes and conjugated diene (Jiang et al., 1992; Milne et al., 2017) could have been used to assess lipid peroxidation hence, future studies are recommended to assess for such markers.

Furthermore, it will be helpful to determine by mass spectrophotometry the form and uptake of zingerone into the liver and evaluate the duration of its residence in the liver before complete excretion. Additionally, it would be interesting to learn more about zingerone's epigenetic remodelling and given its lipophilic nature, it would be important to study zingerone's its impact on adipose tissue and the pancreas.

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LIST OF APPENDICES

APPENDIX I-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 Bernice Asiedu (Student number: 1889383) am a student registered for the degree of Doctor of Philosophy in Physiology in the academic year 2019.

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: 13th January, 2023

APPENDIX II-ANIMAL USE ETHICAL CLEARANCE CERTIFICATE

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2019/10/57/B

APPLICANT: Ms B Asiedu
School: School of PhysiologyN/A
Department: Endocrinology and Metabolism; Location: CAS

PROJECT TITLE: Neonatal orally administered zingerone: potential to programme for protection against ethanol-induced fatty liver disease in Sprague Dawley rats

Category: B Species and Numbers involved: *120X 10-day old female nursing pups, Sprague Dawley Rats, 120X 10-day old male nursing pups, Sprague Dawley Rats and 24X Adult nulliparous female Sprague Dawley Rats*

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 29 Oct 2019. This approval remains valid until 12 Jan 2022.

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

Signed: _____ Date: 14th January 2020
(Chairperson of the AREC)

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

Signed: _____ Date: 14 January 2020

CC: Student supervisor: «Title1» «Initials1» «Supervisor_surname»
Director Central Animals Service: Dr Kim Jardine

APPENDIX III-FIRST MODIFICATIONS & EXTENSIONS TO EXPERIMENT

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: Asiedu, Bernice

b. Department: School of Physiology

c. Experiment to be modified / extended

	AESC NO		
Original AESC number	2019	10	57B
Other M&Es :			

d. Project Title: The potential of zingerone to protect against alcoholic liver disease

		No.	Species
e.	Number and species of animals originally approved:	240	*Sprague Dawley Rats*
f.	Number of additional animals previously allocated on M&Es:		
g.	Total number of animals allocated to the experiment to date:	240	*Sprague Dawley Rats*
h.	Number of animals used to date:		

i. Specific modification / extension requested:

- To reduce the zingerone dosage from 100mg/kg body weight to 40 mg/kg body weight.
- Add two more research assistance/co-workers to assist with the care of animal handling and sample collection. I propose to add the following postgraduate students: Mmahiine Mosana and Bayanda Mdoda

Motivation for modification/extension:

- From previous work done in our laboratory, examiners recommended the use 40mg/kg wt of zingerone which is not only effective but tolerable to rat pups.
- Most of the co-workers on my ethics have submitted their theses/dissertations for examination thus will no longer be available hence the need for replacements who will be able to assist.

AESC 2012 M&E

School of Physiology
Faculty of Health Sciences
University of the Witwatersrand
Parktown Campus, 2193
Johannesburg
South Africa.

2nd June, 2020

Animal Research Ethical Committee, University of the Witwatersrand

Attention: Chairman

Dear Prof Penny,

RE: Responses to assessors' comments on Modification and Extension Application

I would like to thank the Ethics Committee for their positive and valuable feedback on my modification and extension application to my ethics. I have answered the questions raised by the committee to the best of my abilities. Please find below my responses.

Question 1: Is the researcher able to provide a published reference to the research pertaining to the reduced zingerone concentrations?

Mani et al. (2016) administered 40mg/kg zingerone which was subsequently used by Muhammad (2018). Nasiru Muhammed, a Ph. D student in our lab, fed 40mg/kg to rat pups while Mani et al. (2016) fed it (40mg/kg bwt) to adult rats. Importantly, the administration of zingerone at 40mg/kg was found to be effective at preventing non-alcoholic fatty liver disease (Muhammed, 2018). Since I will be administering the zingerone to rat pups, I thought it prudent to use the 40mg/kg

Question 2: Were any adverse events observed in the animals at the 100mg/kg zingerone concentration treatment that warranted the decrease in concentration requested in this M&E?
The administration of zingerone at 100mg/kg bwt was initially selected based on OECD guidelines, where in 1/10th of LD_{50} is considered safe dose for chemopreventive studies (Kumar et al., 2014; Rao et al., 2009). However, this dose had not been tried in rat pups. Besides zingerone has a pungent taste that can activate transient receptor potential cation channel subfamily V member 1 (TRPV1), the receptor for heat and pain (Komai et al., 2006) hence my comfort with the lower dose (40mg/kg) which has been used and demonstrated to be efficacious against NAFLD.

References

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My supervisors' have reviewed and approved the changes made. I hope that all the issues raised have been adequately addressed. I look forward to a positive response.

Yours sincerely,
Bernice Asiedu (1889383)

Date: 15/05/2020

Signature: 

RECOMMENDATIONS

Approval of lower concentration zingerone (40mg/kg) usage for the study. The AREC requests that toxicity is monitored in the rat pups and any adverse events are reported immediately to the committee, as this lower concentration is still yet to be proven as safe in the younger rat pups. Approval of additional co-workers, M. Mosana and M. Mdoda to the study on condition that they attend the CAS-orientation course. Please contact Lorraine Matjila (Lorraine.motjila@wits.ac.za) for booking details.

Date: 04 June 2020

Signature: 

Deputy-Chairman, AESC

APPENDIX IV- SECOND MODIFICATIONS & EXTENSIONS TO EXPERIMENT

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: Asiedu, Bernice

b. Department: School of Physiology

c. Experiment to be modified / extended

			AESC NO	
Original AESC number	2019	10	57B	
Other M&Es : Reduce zingerone dosage from 100mg/kg/bwt to 40 mg/kg/bwt				

d. Project Title: The potential of zingerone to protect against alcoholic liver disease

		No.	Species
e.	Number and species of animals originally approved:	240	*Sprague Dawley Rats*
f.	Number of additional animals previously allocated on M&Es:	N/A	
g.	Total number of animals allocated to the experiment to date:	240	*Sprague Dawley Rats*
h.	Number of animals used to date:	40	

i. Specific modification / extension requested:

To remove saccharin from plain water and alcohol solution at phase 3 of the experiment.

Motivation for modification/extension:
Originally, saccharin(0.025%) was proposed to be added to the drinking solutions (plain water and alcohol) at phase 3 (From PND 46-100) of the experiment to improve palatability of and encourage alcohol consumption by the rats since they (rats) have a natural aversion to alcohol. Saccharin increases food and fluid consumption in rats (Andrejić et al., 2013; Azeez et al., 2019). Additionally, dietary saccharin has been shown to affect hepatic and pancreatic function as a result of increased diameter and volume density of both Langerhans islets and exocrine acini as well as vacuolization of the hepatic cytoplasm(Andrejić et al., 2013). The metabolic effects of saccharin will interfere with the effects of alcohol, thus introduce confounding factors. Researchers (Cano et al., 2003; Ojeda et al., 2008) demonstrated that alcohol can be put in drinking water from 5% with a gradual increment of alcohol concentration to 20% producing ethanol consumption of 5.5, 7.8 and 8.9 g/kg/bwt/day and can induce oxidative stress and inflammation-underpinning mechanisms for ALD. This approach removes the confounding effects of saccharin. Further, another recent publication that used 12% ethanol solution as sole drinking water induced ALD in adults rats (Radic et al., 2019). There is, therefore, no doubt that the method adopted by this protocol will be sufficient to induce ALD without saccharin.

References

Andrejić, B. M., Mijatović, V. M., Samojlik, I. N., Horvat, O. J., Čalasan, J. D., & Đolai, M. A. (2013). The influence of chronic intake of saccharin on rat hepatic and pancreatic function and morphology: Gender differences. *Bosnian Journal of Basic Medical Sciences*, *13*(2), 94–99.

Azeez, O. H., Alkass, S. Y., & Persike, D. S. (2019). Long-Term Saccharin Consumption and Increased Risk of Obesity, Diabetes, Hepatic Dysfunction, and Renal Impairment in Rats. *Medicina (Kaunas,*

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Radic, I., Mijovic, M., Tatalovic, N., Mitic, M., Lukic, V., Joksimovic, B., Petrovic, Z., Ristic, S., Velickovic, S., Nestorovic, V., Corac, A., Miric, M., Adzic, M., Blagojevic, D., Popovic, L., & Hudomal, S. (2019). Protective effects of whey on rat liver damage induced by chronic alcohol intake. *Human & Experimental Toxicology*, *38*(6), 632–645. https://doi.org/10.1177/0960327119829518

Date: 28/08/2020

Signature:

RECOMMENDATIONS

Approval of the modification to omit saccharin from the protocol as described above.

Date:8 September 2020

Signature:

Deputy-Chair, AESC

APPENDIX V- THIRD MODIFICATIONS & EXTENSIONS TO EXPERIMENT

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: Asiedu, Bernice

b. Department: School of Physiology

c. Experiment to be modified / extended

		AESC NO	
Original AESC number	**2019**	**10**	**57B**
Other M&Es : 1. Reduce zingerone dosage from 100mg/kg/bwt to 40 mg/kg/bwt 2. Remove saccahrin from drinking fluid at phase 3 of the xepriment			

d. Project Title: The potential of zingerone to protect against alcoholic liver disease

		No.	Species
e.	Number and species of animals originally approved:	240	*Sprague Dawley Rats*
f.	Number of additional animals previously allocated on M&Es:	N/A	
g.	Total number of animals allocated to the experiment to date:	240	*Sprague Dawley Rats*
h.	Number of animals used to date:	202	

i. Specific modification / extension requested:

To creat an additional group.
To add on new co-workers.

Remmotile Kganya Letsoalo
Nqobile Ndlazi

Motivation for modification/extension:

We would like to add an extra experimental group (10 males and 10 females) which will be administered with distilled water at the neonatal phase. This is to creat a neat control group to nullify any effect the artificial milk might have. The rationale for culling rodent litters to 8-12 as we did in our study is to avoid over-nutrition or under-nutrition (Parra-Vargas et al., 2020). However, supplementing breast-feeding with articial milk formula may lead to over-nutrition in infants (Smith & Becker, 2016). Hence to provide proof that the artificial milk used as vehicle for administrating the drugs (alcohol and zingerone) had no effect, an additional neat experimental will be required.

Additionally, we would like to add more co-workers to assist with technical and animal work especially because the project is on-going during the Christmas and other co-workers have indicated their unavailability.

Remmotile Kganya Letsoalo
Nqobile Ndlazi

AESC 2012 M&E

References

Parra-Vargas, M., Ramon-Krauel, M., Lerin, C., & Jimenez-Chillaron, J. C. (2020). Size Does Matter: Litter Size Strongly Determines Adult Metabolism in Rodents. *Cell Metabolism*, *32*(3), 334–340. https://doi.org/10.1016/j.cmet.2020.07.014

Smith, H. A., & Becker, G. E. (2016). Early additional food and fluids for healthy breastfed full-term infants. *The Cochrane Database of Systematic Reviews*, *8*, CD006462. https://doi.org/10.1002/14651858.CD006462.pub4

Date: 06/11/2020

Signature:

RECOMMENDATIONS

Approval for the additional experimental animal group and the addition of co-workers R. Letsoalo and N. Ndlazi to the study. Please ensure that the newly appointed co-workers report to Dr K. Jardine for orientation.

Date:20 Novemeber 2020 Signature:

Deputy-Chair, AESC

APPENDIX VI- 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 0.5g 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 200ml 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 evaporated 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 (%):

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

Storage:
Add a little petroleum ether (5ml) to the oil and store at 4 ± 2°C.

APPENDIX VIII- 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 VII- 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 IX- SUPPLEMENTARY DATA

Table S3.1: The effect of nutritive milk on tibia and femoral masses, lengths and Seedor indices in suckling male and female pups

Parameter	Sex	DH_20	NM	t-Value	p-value
Induction body mass (g)	Male	24.71±2.33	23.94±2.98	0.56	0.59
	Female	23.88±2.67	22.22±2.14	0.68	0.51
Termination body mass (g)	Male	46.36±9.64	43.50±6.41	1.42	0.18
	Female	47.63±9.23	41.33±8.64	1.45	0.17
Tibia Mass (mg)	Male	60.14±15.15	48.88±9.78	1.74	0.11
	Female	56.75±12.99	46±13.26	1.72	0.11
Tibia length (mm)	Male	16.49±1.45	15.91±1.28	0.83	0.42
	Female	15.78±1.23	15.43±1.22	2.33	0.03
Tibia length/mass (mm/mg)	Male	3.61±0.65	3.05±0.38	2.08	0.06
	Female	3.35±0.58	2.94±0.60	1.45	0.17
Empty Carcass (g)	Male	36.29±6.60	33.50±5.35	0.91	0.38
	Female	36.94±7.60	32.75±7.12	1.20	0.25

Data is presented as mean ± standard deviation. p-value was set at $p < 0.050$; NM = gavaged with 10 mL/kg of nutritive milk. DH_20=Distilled water

Table S3.2: The effect of nutritive milk on oxidative stress biomarkers, visceral fat and hepatic trighlyceride in suckling male and female rat pups

Parameter	Sex	DH_20	NM	t-Value	p-value
CYP2E1(ng/mL)	Male	70.63±22.88	69.97±14.76	0.06	0.95
	Female	75.06±25.72	74.94±17.38	0.01	0.99
Catalase(µmol of H_20_2 consumed)	Male	2.14±0.83	2.14±0.94	0.02	0.98
	Female	2.29±0.84	1.92±0.90	0.70	0.50
SOD(U/mg protein)	Male	7.09±2.69	5.18±2.46	0.39	0.70
	Female	6.55±2.59	5.42±1.36	0.06	0.95
T GSH(µmol/g)	Male	5.88±2.03	4.35±1.81	1.54	0.15
	Female	5.56±1.49	5.77±1.66	0.26	0.80
TBARS(nmol/L)	Male	0.44±0.22	0.38±0.98	0.55	0.59
	Female	0.44±0.14	0.47±0.23	0.27	079
Hepatic Triglyceride (mmol/gprotein)	Male	0.65±0.09	0.60±0.08	1.07	0.30
	Female	0.64±0.10	0.60±0.55	1.16	0.26
Visceral fat (% bwt)	Male	62.84±18.88	50.61±15.27	1.24	0.24
	Female	54.77±11.77	51.25±15.21	0.50	0.62

Data is presented as mean ± standard deviation. p-value was set at $p < 0.050$; NM = gavaged with 10 mL/kg bwt of nutritive milk. DH_20=Distilled water

Table S3.3: The effect of nutritive milk of zingerone on glucose and lipid profile in suckling male and female rat pups.

Parameter	Sex	DH_20	NM	t-value	p-value
Glucose (mmol/l)	Female	5.49±0.39	5.74±0.53	1.12	0.28
	Male	5.59±0.62	5.41±0.37	0.66	0.52
TG(mmol/l)	Female	0.93±0.16	0.85±0.21	0.84	0.41
	Male	0.92±0.28	0.88±0.37	0.23	0.82
HDL (mmol/l)	Female	1.64±0.20	1.63±0.46	0.05	0.96
	Male	2.32±0.52	1.68±0.42	1.99	0.07
TC (mmol/l)	Female	2.33±0.54	2.49±0.76	0.38	0.71
	Male	2.59±0.43	2.66±0.91	0.16	0.87
LDL (mmol/l)	Female	1.32±0.43	1.19±0.96	0.29	0.78
	Male	1.09±0.18	1.35±0.54	1.01	0.34

Data is presented as mean ± standard deviation. p-value was set at $p < 0.050$. DH_20-Distilled water; NM= gavaged with 10 mL/kg of nutritive milk. TG=Triglycerides; HDL-high density lipoprotein cholesterol; TC= Total cholesterol; LDL=Low density lipoprotein cholesterol

Table S3.4: The effect of nutirtive milk on glucose and lipid metabolizing hormones in male and female suckling rat pups

Parameter	**Sex**	DH_20	NM	t-value	p-value
Glucose (mmol/l)	Female	5.49±0.39	5.74±0.53	1.12	0.28
	Male	5.59±0.62	5.41±0.37	0.66	0.52
Insulin (ng/ml)	Female	1.47±0.14	1.10±0.47	2.69	0.02
	Male	1.47±0.26	1.10±0.32	2.19	0.05
HOMA-IR	Female	0.48±0.09	0.37±0.18	1.95	0.07
	Male	0.44±0.11	0.35±0.18	1.20	0.25
Leptin (ng/ml)	Female	1.10±0.38	0.91±0.21	0.68	0.51
	Male	1.10±0.32	0.89±0.27	1.11	0.29

Data is presented as mean ± standard deviation. p-value was set at $p < 0.050$. ab = within row means with different letters significantly different at $P < 0.05$; DH_20-Distilled water; NM-gavaged with 10ml/kg of nutritive milk: HOMAIR = homeostatic model assessment of insulin resistance; ADP = Adiponectin; TG=Triglycerides; HDL =- high density lipoprotein cholesterol; TC = Total cholesterol; LDL = low density lipoprotein cholesterol

Table S3.5: The nutritive milk on the lengths, absolute and relative weights of visceral organs in female suckling rat pups.

Organ	DH_20	NM	t-value	p-value
Heart (g)	0.33±0.05	0.31±0.06	0.99	0.34
Heart rTL	0.20±0.04	0.20±0.02	0.005	0.99
Liver (g)	2.10±0.41	1.80±0.37	1.50	0.15
Liver rTL	1.25±0.17	1.19±0.21	0.64	0.53
Kidney (g)	0.61±0.086	0.55±0.10	1.30	0.21
Kidneys rTL	0.36±0.03	0.36±0.05	0.31	0.76
Stomach(g)	0.47±0.09	0.40±0.07	1.82	0.08
Stomach rTL	0.28±0.04	0.26±0.03	1.09	0.29
Caecum (g)	0.34±0.08	0.28±0.07	1.44	0.17
Caecum rTL	0.20±0.05	0.19±0.05	0.32	0.76
Pancreas (g)	0.25±0.06	0.21±0.04	1.97	0.07
Pancreas rTL	0.15±0.03	0.13±0.03	1.17	0.26
SI (g)	2.78±0.39	2.55±0.39	1.97	0.07
SI (cm)	78.13±3.83	70.70±7.68	2.49	0.02
SI r TL	1.66±0.18	1.55±0.24	1.79	0.07
LI (g)	0.48±0.10	0.36±0.09	2.58	0.02
LI (cm)	11±1.07	10.1±1.63	1.34	0.20
LI Rtl	0.28±0.05	0.24±0.05	1.94	0.07

Data is presented as mean ± standard deviation. p-value was set at $p < 0.050$. ab = within row means with different letters significantly different at $p < 0.050$; DH_20-Distilled water; NM-gavaged with 10ml/kg of nutritive milk; SI = small intestine; LI = large intestine; rTL = weight of organ masses expressed relative to tibial length (g/cm).

Table S3.6: The effect of nutritive milk on the lengths, absolute and relative weights of visceral organs in suckling male pups.

Organ	DH20	NM	t-value	p-value
Heart (g)	0.33±0.052	0.32±0.05	0.40	0.69
Heart rTL	0.20±0.034	0.20±0.01	0.23	0.82
Liver (g)	2.14±0.41	1.93±0.40	1.00	0.33
Liver rTL	1.29±0.184	1.18±0.18	1.22	0.25
Kidney (g)	0.59±0.09	0.55±0.08	0.86	0.41
Kidneys rTL	0.36±0.034	0.35±0.03	0.67	0.51
Stomach(g)	0.46±0.07	0.43±0.08	0.88	0.39
Stomach rTL	0.28±0.04	0.27±0.04	0.55	0.59
Caecum (g)	0.33±0.04	0.30±0.04	0.93	0.06
Caecum rTL	0.19±0.03	0.18±0.03	1.15	0.27
Pancreas (g)	0.23±0.04	0.24±0.05	0.31	0.76
Pancreas rTL	0.14±0.03	0.15±0.03	0.50	0.63
SI (g)	2.70±0.32	2.44±0.49	1.19	0.25
SI(cm)	77.86±7.01	74.29±6.98	0.96	0.36
SI r TL	1.64±0.10	1.53±0.24	1.15	0.27
LI (g)	0.42±0.05	0.40±0.13	0.31	0.76
LI (cm)	10.71±1.66	10.31±1.60	0.48	0.64
LI rTL	0.25±0.02	0.25±0.07	0.17	0.86

Data is presented as mean ± standard deviation. p-value was set at $p < 0.050$. ab = within row means with different letters significantly different at $p < 0.050$; DH20-Distilled water; NM-gavaged with 10ml/kg of nutritive milk; SI = small intestine; LI = large intestine; rTL = weight of organ masses expressed relative to tibial length (g/cm).

Table S4.1: Composition of standard rat chow

Rat Chow Ingredient	g/kg
Protein	220
Moisture	100
Oils and Fats	50
Linoleic acid	12
Fibre	40
Ash	70
Calcium	12
Phosphorus	7.5
Vitamin E	100
	IU/kg
Vitamin A	16000
Vitamin D	2000

Table S4.2: Weekly feed intake in male (A) and female (B) adult rats

A.Males	Total weekly feed Intake (g/100g body mass)							
Treatment Groups	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8
NM+W^{ad}	34.6±3.7^{a}	38.4±3.8^{abc}	41.5±4.1^{a}	41.1±2.7^{a}	42.6±2.4^{a}	40.4±4.3^{a}	42.7±3.4^{a}	38.4±3.6^{a}
NM+Eth+W^{ad}	33.4±3.7^{a}	39.0±6.3^{a}	42.0±8.3^{a}	40.0±9.2^{a}	40.5±7.8^{a}	41.6±4.7^{a}	40.5±4.2^{a}	36.2±5.4^{a}
NM+Eth^{ad}	31.9±3.0^{a}	35.6±4.6^{ab}	27.2±4.6^{b}	30.1±3.4^{b}	32.2±2.6^{b}	31.3±1.1^{b}	30.5±2.8^{b}	27.4±2.7^{b}
NM+Eth+Eth^{ad}	31.5±4.7^{a}	31.6±3.4^{b}	24.8±3.7^{b}	28.3±2.4^{b}	31.9±3.8^{b}	28.4±2.7^{b}	32.9±4.6^{b}	27.6±2.6^{b}
NM+ZO+W^{ad}	35.5±4.5^{a}	40.4±5.6^{a}	43.1±4.2^{a}	42.8±2.6^{a}	42.6±1.6^{a}	41.8±2.5^{a}	42.8±0.9^{a}	36.4±5.8^{a}
NM+Eth+ZO+W^{ad}	34.9±3.6^{a}	39.1±2.4^{a}	40.0±4.1^{a}	41.2±2.7^{a}	43.7±0.9^{a}	41.2±3.2^{a}	40.7±5.1^{a}	38.8±2.2^{a}
NM+ZO+Eth^{ad}	32.2±4.7^{a}	33.5±4.2^{b}	26.0±4.5^{b}	28.7±2.3^{b}	32.1±2.3^{b}	28.7±4.0^{b}	31.6±3.1^{b}	27.6±2.2^{b}
NM+Eth+ZO+Eth^{ad}	30.0±2.5^{a}	31.6±1.7^{c}	25.4±5.9^{b}	28.4±3.9^{b}	32.5±3.9^{b}	30.1±3.4^{b}	31.4±2.0^{b}	27.9±2.4^{b}

B. Females	Total weekly feed intake (g/100g boy mass)							
Treatment Groups	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8
NM+W^{ad}	46.1±5.5^{a}	50.6±6.1^{a}	47.6±6.1^{a}	50.9±8.0^{a}	52.8±9.4^{a}	50.8±6.0^{a}	48.3±3.1^{a}	43.9±3.7^{a}
NM+Eth+W^{ad}	43.5±4.2^{ac}	47.2±4.6^{a}	47.0 ±5.2^{a}	46.9±2.6^{a}	48.4±4.7^{a}	48.1±5.0^{a}	50.6±2.8^{a}	43.0±5.1^{a}
NM+Eth^{ad}	35.0±8.0^{bc}	36.0±5.0^{b}	30.0±7.0^{b}	33.0±5.0^{b}	35.3±6.8^{b}	33.6±7.0^{b}	33.9±5.3^{b}	29.3±4.8^{b}
NM+Eth+Eth^{ad}	36.0±5.0^{bc}	38.2±3.6^{b}	29.0±5.0^{b}	35.0±5.0^{b}	33.5±1.7^{b}	32.2 ±2.0^{b}	31.4±3.7^{b}	30.3±2.8^{b}
NM+ZO+W^{ad}	44.0±3.0^{a}	47.9±4.1^{a}	49.0±6.0^{a}	45.0±6.0^{a}	50.4±3.1^{a}	47.6 ±4.0^{a}	48.3±3.2^{a}	43.0±4.7^{a}
NM+Eth+ZO+W^{ad}	41.0±6.0^{ac}	45.0±4.0^{a}	49.0±6.0^{a}	48.0±4.0^{a}	49.6±3.2^{a}	48.0±5.0^{a}	48.3±4.0^{a}	45.0±4.4^{a}
NM+ZO+Eth^{ad}	39.0±3.0^{ac}	36.4±3.4^{b}	28.0±5.0^{b}	31.0±2.0^{b}	33.9±3.7^{b}	32.5±2.0^{b}	33.4±4.0^{b}	31.1±4.8^{b}
NM+Eth+ZO+Eth^{ad}	36.0±3.0^{bc}	36.9±4.0^{b}	32.0±4.0^{b}	34.0±6.0^{b}	34.6±2.9^{b}	32.6±3.0^{b}	32.1±4.9^{b}	29.8±4.4^{b}

Data presented as mean ± standard deviation. ^{ab} = within row means with different letters significantly different at $p < 0.05$. **NM + W^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk during suckling + plain drinking water in adulthood; **NM+Eth+ W^{ad}** = gavaged with 10 mL/kg body mass per day

nutritive milk and Eth during suckling + plain drinking water in adulthood; **NM+*Eth*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk during suckling + Eth solution in adulthood; **NM + Eth+ *Eth*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk and Eth during suckling + Eth solution in adulthood; **NM+ ZO + *W*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk and ZO during suckling + plain drinking water in adulthood; **NM+Eth+ZO+*W*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk, Eth and ZO during suckling + plain drinking water in adulthood **NM+ZO+*Eth*^{ad}** = gavage with 10 mL/kg body mass per day nutritive milk and ZO during suckling + Eth solution in adulthood; **NM+Eth+ZO+ *Eth*^{ad}** = gavage with 10 mL/kg body mass per day nutritive milk, Eth and ZO during suckling + Eth solution in adulthood. n = 6-8 per treatment group

Table S4.3: Weekly fluid intake in male (A) and female (B) adult rats

A. Males	Fluid intake (mL/100g body mass)							
Treatment Group	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8
NM+W^{ad}	66.8±9.8^{a}	75.5±8.4^{a}	83.2±8.7^{a}	83.7±6.6^{a}	87.4±12.8^{a}	84.1±13.1^{a}	91.6±16.9^{a}	70.6±13.3^{ab}
NM+Eth+W^{ad}	76.02±30.6^{a}	81.9±22.4^{a}	86.2±10.9^{a}	87.3±14.2^{a}	86.5±22.3^{a}	82.3±19.0^{a}	87.2±17.2^{a}	78.6±13.4^{a}
NM+Eth^{ad}	87.2±22.8^{a}	65.5±8.6^{a}	46.7±10.4^{b}	61.8±5.2^{b}	60.7±5.1^{b}	61.7±5.9^{b}	69.4±17.4^{a}	59.2±21.9^{ab}
NM+Eth+Eth^{ad}	66.5±13.4^{a}	68.2±17.6^{a}	45.1±8.9^{b}	52.3±8.8^{b}	54.1±15.4^{b}	49.5±10.6^{b}	50.9±9.6^{b}	51.0±14.9^{b}
NM+ZO+W^{ad}	68.6±18.5^{a}	82.3±9.9^{a}	86.4±5.6^{a}	85.4±12.1^{a}	85.4±9.6^{a}	83.8±10.8^{a}	83.9±9.2^{a}	65.9±15.1^{ab}
NM+Eth+ZO+W^{ad}	72.9±18.3^{a}	79.9±12.1^{a}	82.8±15.0^{a}	85.9±7.4^{a}	85.5±9.1^{a}	82.7±4.9^{a}	81.5±19.0^{a}	73.4±6.2^{ab}
NM+ZO+Eth^{ad}	75.0±22.9^{a}	69.4±14.3^{a}	47.8±11.9^{b}	53.4±6.7^{b}	56.3±10.2^{b}	56.6±9.7^{b}	52.4±15.7^{b}	55.8±20.8^{ab}
NM+Eth+ZO+Eth^{ad}	69.9±10.6^{a}	67.7±18.4^{a}	48.2±11.1^{b}	58.9±12.5^{b}	56.4±9.4^{b}	55.8±9.8^{b}	55.5±10.1^{b}	53.9±11.7^{ab}

B. Females	Fluid intake (mL/100g body mass)							
Treatment Group	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8
NM+W^{ad}	101.9±20.8^{a}	106.2±34.3^{a}	103.2±20.2^{a}	104.8±17.1^{a}	109.1±13.3^{a}	97.9±18.5^{a}	111.6±19.6^{a}	95.3±25.6^{a}
NM+Eth+W^{ad}	104.3±13.4^{a}	105.2±11.1^{a}	104.7±16.4^{a}	107.5±16.8^{a}	94.0±16.6^{a}	96.1±8.5^{a}	109.0±13.9^{a}	98.7±12.8^{a}
NM+Eth^{ad}	95.0±23.0^{a}	84.2±16.7^{a}	62.3±9.4^{b}	70.3±17.8^{b}	75.6±41.9^{a}	68.2±17.0^{b}	67.9±10.1^{b}	66.7±17.5^{a}
NM+Eth+Eth^{ad}	106.8±24.5^{a}	96.9±31.8^{a}	76.8±23.7^{a}	81.1±33.7^{a}	69.4±10.2^{b}	67.5±8.0^{b}	69.4±12.2^{b}	61.1±14.3^{b}
NM+ZO+W^{ad}	105.3±27.3^{a}	118.4±22.5^{a}	110.9±26.7^{a}	120.9±23.1^{a}	120.0±32.1^{a}	119.3±30.9^{a}	116.0±15.9^{a}	102.6±26.9^{a}
NM+Eth+ZO+W^{ad}	100.0±32.0^{a}	98.2±24.2^{a}	108.3±25.1^{a}	109.5±17.9^{a}	111.3±10.6^{a}	102.1±20.3^{a}	103.6±12.2^{a}	86.0±18.0^{a}
NM+ZO+Eth^{ad}	116.0±14.4^{a}	94.8±35.0^{a}	66.9±20.4^{b}	63.4±10.1^{b}	76.34±17.16^{a}	64.5±16.0^{b}	66.5±16.2^{b}	72.2±26.4^{a}
NM+Eth+ZO+Eth^{ad}	113.3±31.7^{a}	96.3±22.9^{a}	61.5±10.1^{b}	68.7±12.4^{b}	71.99±24.5^{b}	66.2±7.2^{b}	62.0±15.7^{b}	63.2±13.1^{b}

Data presented as mean ± standard deviation. ^{ab} = within column means with different letters significantly different at $p < 0.05$. **NM + W^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk during suckling + plain drinking water in adulthood; **NM+Eth+ W^{ad}** = gavaged with 10

mL/kg body mass per day nutritive milk and Eth during suckling + plain drinking water in adulthood; **NM+*Eth*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk during suckling + Eth solution in adulthood; **NM + Eth+ *Eth*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk and Eth during suckling + Eth solution in adulthood; **NM+ ZO + *W*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk and ZO during suckling + plain drinking water in adulthood; **NM+Eth+ZO+*W*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk, Eth and ZO during suckling + plain drinking water in adulthood; **NM+ZO+*Eth*^{ad}** = gavage with 10 mL/kg body mass per day nutritive milk and ZO during suckling + Eth solution in adulthood; **NM+Eth+ZO+ *Eth*^{ad}** = gavage with 10 mL/kg body mass per day nutritive milk, Eth and ZO during suckling + Eth solution in adulthood n = 6-8 per treatment group

Table S4.4: Weekly calorie intake of male (A) and female (B) adult rats

A. Males	Total weekly calories intake (Kcal/100g body mass)							
Treatment Groups	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8
NM+W^{ad}	97±10.3^{a}	107.4±10.7^{a}	116.3±11.3^{a}	115.1±7.61^{a}	119.4±6.6^{a}	113.2±12.0^{a}	119.6±9.6^{a}	107.5±10.0^{a}
NM+Eth+W^{ad}	93.4±10.3^{a}	109.3±17.7^{a}	117.7±23.2^{a}	111.9±25.8^{a}	113.3±21.9^{a}	116.6±13.2^{a}	113.5±11.8^{a}	101.3±15.1^{a}
NM+Eth^{ad}	114.0±13.0^{a}	136.0±14.0^{b}	128.6±21.5^{a}	154±10.9^{b}	158.3±7.9^{b}	157.2±7.8^{b}	152.1±22.5^{b}	148.8±12.3^{b}
NM+Eth+Eth^{ad}	107.0±14.0^{a}	126.0±13.0^{a}	120.3±17.8^{a}	138±14.0^{b}	150.3±23.0^{b}	135.3±15.4^{b}	149.5±16.6^{b}	138.7±15.4^{b}
NM+ZO+W^{ad}	99.0±12.0^{a}	113.0±15.0^{a}	120.6±11.9^{a}	119.7±7.2^{a}	119.3±4.4^{a}	117.2±7.0^{a}	119.9±2.8^{a}	101.8±16.1^{a}
NM+Eth+ZO+W^{ad}	97.0±9.0^{a}	109.0±60.0^{a}	111.9±11.5^{a}	115.4±7.6^{a}	122.4±2.6^{a}	115.4±8.9^{a}	113.9±14.1^{a}	108.6±6.2^{a}
NM+ZO+Eth^{ad}	111.0±18.0^{a}	132.0±13.0^{b}	126.6±18.1^{a}	140.6±10.7^{b}	153.2±12.3^{b}	144.2±14.5^{b}	151.4±25.6^{b}	143±14.5^{b}
NM+Eth+ZO+Eth^{ad}	103.0±7.0^{a}	126.0±12.0^{a}	125.4±26.1^{a}	145.7±17.9^{b}	154.5±12.0^{b}	147.2±15.6^{b}	148.4±13.8^{b}	143.9±12.8^{b}

B. Females	Total weekly calories intake (Kcal/100g body mass)							
Treatment Groups	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8
NM+W^{ad}	129.2±15.5^{a}	141.7±17.2^{a}	133.3±2^{a}	142.5±22.4^{a}	147.9±26.4^{a}	142.2±18.5^{a}	119.6±9.6^{a}	123±10.5^{a}
NM+Eth+W^{ad}	121.9±11.6^{a}	132.3±12.8^{a}	131.5±14.5^{a}	131.2±7.3^{a}	135.4±13.2^{a}	134.7±15.0^{a}	113.5±11.8^{a}	120.4±14.2^{a}
NM+Eth^{ad}	127.3±24.8^{a}	150.8±15.3^{a}	154.9±25.7^{a}	173.1±24.5^{a}	184±51.3^{a}	170.9±27.0^{b}	152.1±22.5^{b}	165.2±17.8^{b}
NM+Eth+Eth^{ad}	131.3±21.8^{a}	161.5±25.4^{a}	152.6±12.7^{a}	189.3±36.0^{b}	172±12.5^{a}	166.1±8.4^{a}	149.5±16.6^{a}	173.2±11.5^{b}
NM+ZO+W^{ad}	125±9.6^{a}	134.2±11.4^{a}	138.2±18.1^{a}	127.6±18.1^{a}	141±8.8^{a}	133.2±11.2^{a}	119.9±2.8^{a}	120.4±13.1^{a}
NM+Eth+ZO+W^{ad}	117±18.3^{a}	128.3±12.3^{a}	139.9±17.9^{a}	135.2±11.5^{a}	138.8±9.0^{a}	134.5±16.3^{a}	113.9±14.1^{a}	126±12.3^{a}
NM+ZO+Eth^{ad}	142.1±10.6^{a}	155.1±23.1^{a}	154.1±30.2^{a}	159.3±16.3^{a}	181±26.7^{a}	163.5±21.6^{a}	151.4±25.6^{b}	174.3±18.2^{b}
NM+Eth+ZO+Eth^{ad}	135±17.3^{a}	157.5±14.0^{a}	160.9±20.4^{a}	173±17.0^{a}	177.8±24.5^{a}	165.8±13.4^{a}	148.4±13.8^{a}	168.4±16.0^{b}

Data presented as mean ± standard deviation. ^{ab} = within column means with different letters significantly different at $p < 0.05$. **NM + W^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk during suckling + plain drinking water in adulthood; **NM+Eth+ W^{ad}** = gavaged with 10

mL/kg body mass per day nutritive milk and Eth during suckling + plain drinking water in adulthood; **NM+*Eth*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk during suckling + Eth solution in adulthood; **NM + Eth+ *Eth*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk and Eth during suckling + Eth solution in adulthood; **NM+ ZO + *W*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk and ZO during suckling + plain drinking water in adulthood; **NM+Eth+ZO+*W*^{ad}** = gavaged with 10 mL/kg body mass per day nutritive milk, Eth and ZO during suckling + plain drinking water in adulthood; **NM+ZO+*Eth*^{ad}** = gavage with 10 mL/kg body mass per day nutritive milk and ZO during suckling + Eth solution in adulthood; **NM+Eth+ZO+ *Eth*^{ad}** = gavage with 10 mL/kg body mass per day nutritive milk, Eth and ZO during suckling + Eth solution in adulthood n = 6-8 per treatment group

Table S5.1: Effect of neonatal zingerone on ethanol consumption in alcohol-exposed rats in adult (A) male and (B) female rats

A. Males					
	Weekly ethanol consumption (g/100g/body mass)				
Week	NM+Eth	NM+Eth+Eth	NM+ZO+Eth	NM+Eth+ZO+Eth	p-value
Wk 1	3.46±0.91[a]	2.64±0.53[a]	2.98±0.91[a]	2.77±0.42[a]	0.189
Wk 2	5.20±0.68[b]	5.41±1.40[b]	5.50±1.13[b]	5.37±1.46[b]	0.974
Wk 3	7.40±1.64[b]	7.16±1.42[b]	7.59±1.89[b]	7.64±1.77[b]	0.94
Wk 4	9.81±0.82[b]	8.30±1.40[b]	8.48±1.07[b]	9.35±1.99[b]	0.174
Wk 5	9.63±0.81[b]	8.59±2.44[b]	8.94±1.61[b]	8.95±1.48[b]	0.745
Wk 6	9.79±0.9[b]	7.86±1.68[b]	8.99±1.54[b]	8.85±1.55[b]	0.14
Wk 7	9.39±3.47[b]	8.09±2.37[b]	8.86±3.30[b]	8.55±1.85[b]	0.848
Wk 8	10.16±1.18[b]	8.68±1.38[b]	9.26±1.49[b]	9.25±1.32[b]	0.278
Mean	8.10±2.52	7.02±2.09	7.57±2.22	7.59±2.34[b]	0.400
A. ***Females***					
	Weekly ethanol consumption (g/100g/body mass)				
Week	NM+Eth	NM+Eth+Eth	NM+ZO+Eth	NM+Eth+ZO+Eth	
Wk 1	3.79±0.94[a]	4.15±1.01[a]	4.61±0.57[a]	4.50±1.26[a]	0.344
Wk 2	6.68±1.32[b]	8.06±2.47[b]	7.52±2.78[b]	7.64±1.82[b]	0.654
Wk 3	9.89±1.49[b]	12.53±8.81[b]	10.62±3.24[b]	9.76±1.60[b]	0.645
Wk 4	11.16±2.82[b]	13.43±5.53[b]	10.05±1.60[b]	10.90±1.96[b]	0.252
Wk 5	11.99±6.65[b]	10.72±1.51[b]	12.11±2.7[b]	11.42±3.88[b]	0.917
Wk 6	10.81±2.70[b]	10.37±0.90[b]	10.23±2.54[b]	10.51±1.14[b]	0.947
Wk 7	10.58±2.77[b]	9.21±1.95[b]	11.46±4.18[b]	10.04±2.07[b]	0.508
Wk 8	11.72±1.74[b]	12.49±1.81[b]	12.31±1.55[b]	11.97±1.93[b]	0.83
Mean	9.58±2.87	10.12±3.01	9.86±2.61	9.59±2.44	

Data is presented as mean ± standard deviation. [ab] = preceding week's ethanol intake was significantly lower at $p < 0.05$. **NM + W** = gavaged with 10 ml/kg body mass per day nutritive milk during suckling + plain drinking water in adulthood; **NM+Eth+ W** = gavaged with 10 ml/kg body mass per day nutritive milk and Eth during suckling + plain drinking water in adulthood; **NM+Eth** = gavaged with 10 ml/kg body mass per day nutritive milk during suckling + Eth solution in adulthood; **NM + Eth+ Eth** = gavaged with 10 ml/kg body mass per day nutritive milk and Eth during suckling + Eth solution in adulthood; **NM+ ZO + W** = gavaged with 10 ml/kg body mass per day nutritive milk and ZO during suckling + plain drinking water in adulthood; **NM+Eth+ZO+W** = gavaged with 10 ml/kg body mass per day nutritive milk, Eth and ZO during suckling + plain drinking water in adulthood; **NM+ZO+Eth** = gavage with 10 ml/kg body mass per day nutritive milk and ZO during suckling + Eth solution in adulthood; **NM+Eth+ZO+ Eth** = gavage with 10 ml/kg body mass per day nutritive milk, Eth and ZO during suckling + Eth solution in adulthood n = 6-8 per treatment group

Table S5.2: Primer sequences

Primer Name	Sequence 5'→3'
SREBP-1C F	CGACACCACCAGCATCAACCACG
SREBP-1C R	GCAGCCCDATTCATCAGCCAGACC
BETAACTIN F	GCTAACAGTCCGCCTAGAAGCA
BETAACTIN R	GTCATCACCATCGGGCAATGAG
PPAR-α F	GATACCACTATGGAGTCCACGCA
PPAR-α R	GCCGAAAGAAGCCCTTG
NF-Kβ F	CGTGAAGTATTCCCAGGTTTG
NF-Kβ R	TGGGGGAAAACTCATCAAAG
TNF-α F	GAAGTTCCCAAATGGCCTCC
TNFα R	GTGAGGGTCTGGGCCATAGA