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ROLE OF HIGH-FAT DIET AND COCOA BEAN EXTRACT ON ADIPOSE TISSUE AND LIVER MORPHOLOGY

BY

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DECLARATION

DECLARATION BY CANDIDATE

I hereby declare that except for references to work of other researchers, which have been duly referenced, this project is the product of my own research carried out under supervision in accordance with regulations of the School of Research and Graduate Studies, University of Ghana. I further declare that this dissertation has neither in whole nor in part been presented for another degree elsewhere, and that I am solely responsible for any residual flaws in this work.

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DECLARATION BY SUPERVISORS

We declare that the practical work and presentation of this thesis were supervised by us in accordance with guidelines on supervision of thesis laid down by the University of Ghana.

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DEDICATION

I dedicate this work to my Lord and Personal Saviour Jesus Christ for His grace and protection for making this come to light. To my parents Mr & Mrs Kingdoh for their relentless pursuit to my achievements in Academia. Also to my dearest wife Jacqueline Kingdoh for her physical and spiritual support. Finally to my children Ekua Gyamfua, Nyameyeodo and Nana Ampong Kwesi. May the Almighty God protect and bless you in all endeavours in life.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
HFD:	High-fat Diet
NAFLD:	Non-alcoholic Fatty Liver Diseases
CVD:	Cardiovascular Diseases
IR:	Insulin Resistance
T2DM:	Type 2 Diabetes Mellitus
AT:	Adipose Tissue
VAT:	Visceral Adipose Tissue
SAT:	Subcutaneous Adipose Tissue
WAT:	White Adipose Tissue
BAT:	Brown Adipose Tissue
ROS:	Reactive Oxygen Species
MCP-1:	Monocyte Chemo-attractant Protein 1
TNF-α:	Tumor Necrosis Factor alpha
IL-1β:	Interleukin 1beta
JNK:	c-Jun N-terminal Kinase
NF-KB:	Nuclear Factor kappa B

LDL:	Low Density Lipoprotein
CCK:	Cholecystokinin
ATM:	Adipose Tissue Macrophages
OGTT:	Oral Glucose Tolerance Test
FBG:	Fasting Blood Glucose
$CD8^{+}$:	Clusters of Differentiation
IKKB:	Inhibitor of kappa B kinase β
IKB:	Inhibitor of kappa B
TLR:	Toll-like Receptors
CCL2:	C-C Motif Chemokine Ligand 2
CXCL5:	C-X-C Motif Chemokine Ligand 5
PI3K:	Phosphatidylinositol 3-Kinase
MAPK:	Mitogen activated Protein Kinase
Nrf2:	Nuclear Factor Erythroid 2
ERK:	Extracellular signal-Regulated Kinase
GSK3:	Glycogen Synthase Kinase 3
PEPCK:	Phosphoenolpyruvate Carboxykinase
AMPK:	Adenosine Monophosphate Activated Protein Kinase

ABSTRACT

Background: Excessive intake of fat is a risk factor for obesity and results in remodeling and dysfunction of adipose tissue with subsequent damage to the liver, pancreas, skeletal muscles, and blood vessels. In spite of several public health initiatives, high-fat diet-associated complications continue to be an important source of morbidity and mortality across the globe. Over time, obesity and adipose tissue dysfunction develop with subsequent deposition of fat droplets into the liver cells (liver steatosis). This obesity-associated remodeling of the adipose tissue and the liver cells generate a systemic pro-inflammatory state mediated by the production of adipokines such as TNF-α, IL-1β and IL-6 which leads to insulin resistance and ultimately contribute to the development of cardiovascular diseases and type 2 diabetes. Cocoa polyphenols have the capacity to restore changes in adipocytes and hepatocytes and improve blood glucose and insulin sensitivity.

Aim: To investigate the role of high-fat diet on inflammation and morphology of adipose tissue and liver cells and whether regular intake of natural cocoa can minimize the effects.

Methodology: Twenty-four (24) male Sprague Dawley rats were randomly assigned into four (4) groups of six (6) animals. Group 1 (control) was the untreated group and was given standard rat chow and tap water. Group 2 was treated with standard rat chow and natural cocoa drink. Group 3 was treated with high-fat diet (40% fat) and tap water. Group 4 was treated with high-fat diet (40% fat) and natural cocoa drink. The experimental period for the treatment was nine (9) weeks. The weight of the animals as well as fasting blood glucose (FBG) were monitored weekly. Blood samples were taken via cardiac puncture before and after the main study, for determination of inflammatory marker, tumor necrosis factor-alpha (TNF-α). The adipose tissue (epididymal, intestinal and mesenteric) and the liver were harvested at the end of the treatment for micro architecture examination and morphometric analysis by stereological methods

Results: High-fat diet resulted in significant increase in weight at the end of 9 weeks, whereas natural cocoa intake reduced this effect (p = 0.0167). The epididymal, intestinal and mesenteric adipocyte sizes increased significantly in HFD only group with average sizes 71.06µm, 56.36µm and 56.17µm respectively compared to all the other groups. Adipocyte size however, was significantly reduced in HFD fed rats treated with cocoa with average epididymal size (60.63µm), intestinal (49.07µm) and mesenteric (48.20µm). The volume density of fat droplets in the hepatocytes was significantly high in rats fed HFD only (4.15×10^2 µm^3) when compared to control and other groups with p=0.0011. Meanwhile rats treated with HFD and cocoa showed significantly lower fat infiltration (0.8×10^2 µm^3) in the hepatocytes. Blood glucose levels increased significantly in high-fat diet only group but were significantly lower in the cocoa fed groups.

Conclusion: Regular High-fat diet intake resulted in increased weight gain, increased adipocyte size, fat infiltration of hepatocytes as well as increased blood glucose levels but natural cocoa intake minimized these effects.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

The regular intake of a high-fat diet (HFD) in individuals and animals is a known risk factor for obesity and other disorders such as insulin resistance, cardiovascular diseases and type 2 diabetes (Bhandari et al, 2011). There is a positive correlation between the level of fat in diet and body weight and it has been established that rats feeding on foods containing high amount of fat gain weight more rapidly than those on a diet containing minimal amount of fat (Hariri & Thibault, 2014). Regular consumption of dietary fat has been shown to be accountable for the rise in adiposity (Hariri & Thibault, 2014). Bray & Popkin, (1998); Schrauwen & Westerterp, (2000) reported that diets rich in fat (≥ 30% of energy from fat) may certainly trigger obesity and its comorbidities such as insulin resistance, cardiovascular diseases and type 2 diabetes.

Adipose tissue has the capacity to store free fatty acids (FFA) efficiently (van Herpen & Schrauwen-Hinderling, 2008). However, the storage capacity of the adipose tissue is exceeded in obese state and free fatty acids begin to accumulate in metabolic tissues such as skeletal muscle, liver and pancreas, initiating lipotoxicity-the accumulation of free fatty acids in ectopic tissues resulting in local inflammation and insulin resistance (Longo *et al*., 2019; van Herpen & Schrauwen-Hinderling, 2008). Excess free fatty acids in turn can enhance inflammatory pathways and impair normal cell signaling within immune cells, adipose tissue, liver and muscle, setting off cellular dysfunction such as alterations in adipokines secretion and insulin resistance (Krahmer *et al.*, 2013).

Obesity may be defined as a body mass index (BMI) greater than 30kg/m² (Gregor & Hotamisligil, 2011). The occurrence of obesity may be due to chronic energy imbalance in which energy intake surpasses energy expenditure resulting in weight gain over time. The additional energy is deposited in body tissues when energy intake surpasses energy expenditure (Anderson *et al.*, 2015). The problem of obesity, encompasses several organ systems and disorders comprising derangements of bodyweight, adipose tissue, metabolism of glucose, levels of cholesterol and inflammatory markers and accumulation of fat in the liver as well as other tissues (Na *et al.*, 2017). Over-nutrition results in obesity and relates to persistent low-grade inflammation (meta-inflammation) in the metabolic tissues including the liver and adipose tissue throughout the body (Lyons *et al.*, 2016).

Obesity and related metabolic syndromes including insulin resistance (IR), type 2 diabetes (T2D) and non-alcoholic fatty liver diseases (NAFLD) are manifested by a state of inflammation instigated by nutrient overload (Grander *et al.*, 2016). Non-alcoholic fatty liver diseases prevalence is fast increasing and is consistent with the increased widespread of obesity worldwide (Younossi, 2019; Olusegun *et al.*, 2012). It is presently the most common chronic liver disease and has become a major liver ailment in children owing to increased prevalence of childhood obesity (Takahashi & Fukusato, 2010).

Obesity is a key issue of public concern in that it affects all stages of life including children and the aged and various socioeconomic groups (Di Cesare et al., 2019). Prevalence of obesity across the world has increased more than 200% since 1980 with nearly 2 billion adults estimated to be overweight in 2014 including 600 million individuals who were obese (Ofori-Asenso *et al.*, 2016). It has been projected that over 115 million individuals suffer from obesity associated complications (Adela Hruby *et al.*, 2015).

The prevalence of overweight in 1990 was highest in the Northern Africa (7.5%) followed by Southern Africa (6.4%), Eastern Africa (4.5%), Middle Africa (3.7%) and Western Africa being the lowest with 2.6% (Ofori-Asenso et al., 2016). There has been an alarming increase of overweight in Southern African region since 1990 with average prevalence rate of 21% in 2015 compared to other regions (Ofori-Asenso *et al*., 2016). Northern African region has also experienced rapid increase in overweight since 1990 with prevalence of 13% in 2015 (Biadgilign *et al*., 2017; Ofori-Asenso *et al*., 2016). It is estimated that as much as 20-50% of urban populations in Africa are either overweight or obese and by 2025, three quarters of the obese population worldwide will be in non-industrialized countries (Amoah, 2003; Puoane *et al*., 2002). In Africa, South Africa is among the countries with the highest prevalence (29% among men and 56% among women) while the Gambia has one of the lowest with an overall prevalence of about 4.0% (Prentice, 2006; Puoane et al., 2002).

In Africa the number of children who are overweight or obese has nearly doubled from 5.4 million in 1990 to 10.6 million in 2014 (Biadgilign *et al*., 2017). Notably, the incidence of childhood obesity has increased in the previous three decades, predisposing them to several health complications affecting their educational attainment and quality of life (Hurt *et al.*, 2010).

Reports indicate that nearly 43% of Ghanaian adults are either overweight or obese. The overall occurrence of overweight and obesity in Ghana is about 23.4 and 14.1% respectively among adults aged 25 years and above and are common among females, elderly, and urban dwellers (Amoah, 2003). The Ghana Demographic and Health Surveys (GDHS) from 1993 to 2014 reported an increasing prevalence of obesity among Ghanaian women (15-49 years) from 3.4% to 15.3% respectively (Ofori-Asenso et al., 2016). Studies show that the prevalence of obesity has remained persistently high in Accra which is the most urbanized region in Ghana whereas in the three

Northern regions as well as Volta region, the prevalence is lowest (Ofori-Asenso *et al*., 2016; Amoah, 2003).

Obesity induces insulin resistance which is a principal risk factor for type 2 diabetes, hyperlipidemia, cardiovascular diseases, and some types of cancer resulting in health complication and death (Park *et al.*, 2014). It has been reported that mice exposed to diet high in fat develop signs related to type 2 diabetes, including insulin resistance and hyperinsulinemia (De MagalhÃes *et al.*, 2019). The progression of local and systemic insulin resistance (IR) come as a result of inflammation particularly in the white adipose tissue (Lackey & Olefsky, 2016).

Inflammation in the adipose tissue has been linked to the development of obesity-associated diseases like cardiovascular diseases, liver steatosis, type 2 diabetes, and insulin resistance (Ormazabal *et al.*, 2018). Adipose cells enlarge in obese individuals and attract immune cells generating an inflammatory setting (Stolarczyk, 2017). Immune cells that are resident undergo both pro-inflammatory and metabolic modification in their role. Inflammatory mediators comprising tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) are triggered by saturated fatty acid and interrupt signaling of insulin (Lyons *et al.*, 2016).

Obesity-induced inflammation comprise high manifestation and production of pro-inflammatory mediators by adipose tissue, skeletal muscles, and liver (Brodmerkel *et al.*, 2005); circulation of pro-inflammatory proteins in elevated concentrations, including cytokines and chemokines and the instigation of pathways that regulate inflammation such as c-Jun N-terminal kinase (JNK) and nuclear factor kappa B cells (NF-kß) pathways (Cai *et al.*, 2006). Studies by Weisberg *et al.*, (2003) suggest that obesity enhances the deposition of macrophages in adipose tissue devoid of varying the macrophage content in liver or muscles. Some of the pro-inflammatory mediators released by

adipose tissues are produced by macrophages and have been involved in the progression and maintenance of obesity-initiated adipose tissue inflammation (Curat *et al.*, 2004).

Non-alcoholic fatty liver (NAFLD) disease is a disorder in which additional fat accumulates in the liver of a patient without a history of alcohol abuse. According to Younossi *et al*. (2016), global prevalence of NAFLD from 22 countries is 25.24% with utmost occurrence in the Middle East and Africa being the least. The incidence of NAFLD in the United States has been reported to be around 10-30% of the total population with similar rates found in Europe and Asia (Farrell *et al.*, 2013). With increasing consumption of diets high in fat in Africa, it can be estimated that an increase in NAFLD may be imminent. The etiology of NAFLD is interrelated to wide-range hepatic insulin resistance, and is viewed as a hepatic expression of metabolic disorder (Paschos & Paletas, 2009).

The mechanism of liver damage in NAFLD is well-thought-out to be a "multiple-hit process" with the first resulting in an increase in fat infiltration of the liver, leading to inflammation *(*Anania *et al,* 2018). Accumulation of triglyceride in the liver, concomitant with insulin resistance is the primary indication of NAFLD which is affected by high energy foods, inactive way of life, and genetic vulnerability (Riazi *et al.*, 2019). Amassing of fat in the liver is accompanied with lipotoxic hepatocellular injury owing to increased free fatty acids, free cholesterol level and additional lipid metabolites (Mendez-Sanchez *et al.*, 2018).

High-fat diet intake has been postulated to perform a critical task in the visceral adipose tissue leading to their expansion, and increases the generation of reactive oxygen species (Jimoh *et al.*, 2018). The secretion of inflammatory adipokines may be due to the stimulation of reactive oxygen species (ROS) resulting in the occurrence of oxidative stress (Otani, 2011). According to Furukawa *et al.,* (2004), one of the primary events in the progression of metabolic disorder in obesity is the

accumulation of ROS in adipose tissues. It has been postulated that calorie restriction leading to weight loss, intake of antioxidant-containing products such as natural cocoa bean, fruits and vegetables as well as regular exercises attenuate the condition of oxidative stress (Imayama *et al.*, 2012). This suggests that regular intake of antioxidant-rich cocoa may inhibit the oxidative stress in adipose tissue and liver and possibly play a role in reducing adipose tissue dysfunction.

Without any restorative pharmacological therapies, lifestyle adjustments involving dietary variations and exercise remain the only effective approach to fight NAFLD and adipose tissue-induced insulin resistance (Riazi *et al.*, 2019). It has been suggested that the best technique to fight the prevalence of obesity is to decrease the calories in foods, with the anticipation that, decreasing the caloric content per unit of food will lead to lower intake of total calories (Swithers *et al.*, 2011).

The cocoa bean is a fruit extensively recognized as one of the high sources of phenolic compounds with the maximum flavanols content of all foods on a per weight basis (Martín & Ramos, 2016). The importance of cocoa flavanols as bioactive compounds with prospective benefits in the prevention of chronic diseases concomitant with inflammation, oxidative stress and metabolic syndromes is still growing (Flores, 2019)**.** Flavanols have the potential of scavenging free radicals as well as its ability to protect tissues from damage (Matsui et al., 2005). A number of studies conducted in the Anatomy Department of University of Ghana Medical School provide evidence that supports the health benefits of cocoa in animals with structural protection against arterial and venous damage and atherosclerotic plaques in progenies of hypercholesterolemia rabbit mothers (Blay *et al.*, 2019). The consumption of cocoa attenuates or prevents disorders including obesity, inflammation, NAFLD, insulin resistance, oxidative stress, hypertension, endothelial dysfunction, and type 2 diabetes (Arora *et al.*, 2015). This study seeks to further investigate the role of high-fat diet induced obesity in adipose tissue dysfunction and how that affects insulin resistance and the

morphology of the liver. It also seeks to assess whether cocoa can reduce morphological damages to the liver and adipose tissue in experimental high-fat diet induced obesity conditions.

1.2 Problem of statement

Ghana ranks the top among forty eight (48) Africa countries with an alarming rise in people living with diabetes mellitus and obesity with estimates at over 23.4 and 14.1% respectively among adults aged 25 years and above and are common among females, elderly, and urban dwellers (Gatimu *et al.*, 2016 & Amoah, 2003).

While the mechanisms underlying adipose tissue, liver tissues and inflammatory changes occurring systematically are known to be mediated via inflammatory markers, little is known how structural aberrations in these tissues as a result of the biochemical and pathological sequalae occurring. Disruption of adipose and liver tissue morphology and function have been implicated in the pathophysiology of diseases concomitant with high-fat intake (Pestana *et al.*, 2017). There is the need to understand the structural modifications and mechanisms by which HFD induces inflammation in the adipose tissue and liver.

This study seeks to provide evidence for understanding the structural changes in the adipose tissue and the liver as well as inflammation that underlie obesity related complications and the likely beneficial effects of antioxidant rich natural cocoa.

1.3 Justification

The increasing trend of high-fat diet intake has been a public health issue raised by clinicians, dietitians and other health workers (Shook *et al.*, 2014). In spite of several public health initiatives, high-fat diet-associated complications continue to be an important source of morbidity and mortality across the globe (Duprez, 2012). It is known that excessive formation and accumulation of reactive oxygen species (ROS) from the metabolism of fat set forth a cascade of events comprising the recruitment of pro-inflammatory mediators resulting in obesity and associated complications (Fernández-Sánchez *et al.*, 2011). Accumulation of ROS from metabolism of high-fat diet in adipose and liver tissues ensues in that the natural over-all antioxidant capacity is compromised when the body especially the liver and adipose are inundated with free radicals (Li *et al.*, 2015). It is therefore feasible to expect that the removal of these free radicals would reduce their damaging effect and a possible attenuation of obesity-related complications.

Cocoa is a rich source of antioxidants owing to its high content of flavanols. The consumption of cocoa eliminate free radicals resulting from metabolism of high-fat diet which prevents several health disorders relating to the liver and adipose tissue including obesity, inflammation, NAFLD, insulin resistance, oxidative stress (Arora *et al.*, 2015).

This work will provide experimental evidence and data to improve the understanding of the mechanism by which high-fat intake leads to obesity, enlarged adipocyte sizes, liver steatosis, diabetes, cardiovascular diseases and how this mechanism can be exploited to develop novel prevention and therapeutic strategies.

1.4 Aim

To investigate the effect of high-fat diet on inflammation and morphology of adipose tissue and liver cells and whether regular intake of natural cocoa can minimize these effects.

1.5 Specific Objectives

- To determine morphological changes in adipocytes in rats treated with high-fat diet.
- To determine morphological changes in hepatocytes (thus fat droplet infiltration) in rats treated with high-fat diet.
- To determine the protective effect of natural cocoa on liver and adipose tissues of high-fat diet fed rats.
- To determine the effect of high-fat diet on blood glucose levels in rats treated with high-fat diet and cocoa.
- To assess effect of natural cocoa on serum levels of inflammatory marker, tumor necrosis factor alpha (TNF-α).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Anatomy of adipose tissue (AT)

Adipose tissue (AT) is a specific type of connective tissue in which adipocytes dominate (Peirce *et al.*, 2016). The adipocytes are found sequestered or in clusters within loose or irregular connective tissue, frequently in large collections where they are the main constituent of adipose tissue. The adipocytes are located throughout the body (Mescher, 2016). Adipose tissue originates from the embryonic mesoderm. The adipose tissue consists of a substantial volume of tissues, amounting to approximately 24L in adult life (Parker, 2018). Obesity corresponds to increased and enlarged adipose tissue exceeding 80L in the morbidly obese individuals (Gómez-Hernández *et al*., 2016)

Adipose tissue is categorized as subcutaneous or visceral, depending on its anatomical location and it is distributed in different proportions throughout the body (Shuster *et al.*, 2012). It is known that expansion of subcutaneous adipose tissue (SAT) has minor or decreased risk of metabolic dysfunction whereas enlarged visceral adipose tissue is strongly associated to metabolic dysfunction (Shuster *et al.*, 2012). Visceral adipose tissue (VAT) in obesity is more prone to become inflamed, and consistently has a stronger influence on systemic inflammation and an aggressive metabolic profile (Pou *et al.*, 2007). Adipose tissue may further be defined into white adipose tissue (WAT) functioning as energy-storing and brown adipose tissue (BAT), the energy-dissipating tissue (Calderon-Dominguez *et al*., 2016). A third type called beige adipose tissue are newly defined type of adipocyte that coexist with white adipocytes in subcutaneous WAT and with brown adipocytes in BAT. Beige adipose tissue shows functional and morphological features as

the brown adipose tissue (Zoico et al., 2019). Brown adipose tissue (BAT) is mostly found in infants and around the clavicle of adults which is specialized in maintaining body temperature (Gao *et al.*, 2015). Several studies show that BAT plays critical role in regulating glucose and triglyceride metabolism (Gunawardana & Piston, 2012; Bartelt *et al.*, 2011).

White adipose tissue (WAT) is found in both subcutaneous and visceral regions. In rodents, the white adipose tissues that are commonly used in studies are inguinal WAT (IWAT), retroperitoneal WAT (RWAT) located behind the kidney (Hung *et al.*, 2014). The rest are intestinal, omental and mesenteric WAT (MWAT) located in the intestinal region around digestive organs and gonadal WAT (GWAT). The gonadal WAT (male epididymal WAT, female parametrial and periovarian WAT) enlarges through the initial periods of obesity (van Beek *et al.*, 2015), serving as a depot for triglyceride storage (Imayama *et al*., 2012). White adipose tissue (WAT) is the long term storage organ that collects excess energy as triglycerides within its lipid droplets, assembling them when needed through the regulation of lipogenesis and lipolysis respectively (Peirce *et al.*, 2016). The white adipose tissue is also an endocrine organ that secretes pro-inflammatory and anti-inflammatory cytokines, hormones, and growth factors, collectively termed adipokines (Lacy *et al.*, 2016). In animals with obesity, WAT deposits increase as a result of hyperplasia and hypertrophy of their adipocytes. Hyperplasia defines the increase in the number of adipocytes which is the healthy expansion of adipocytes resulting in the protection of an organism against metabolic complications (Longo *et al.*, 2019). Hypertrophy on the other hand is the unhealthy expansion of adipocytes promoting obesity-associated metabolic complications (Longo *et al*., 2019; Fuster *et al.*, 2016; Gao *et al.*, 2015).

Nevertheless, when inflammation and obesity are sustained, the adaptive mechanisms that enable adipose tissue to store excess fat fail and result in WAT dysfunction (Pellegrinelli, Carobbio, &

Vidal-puig, 2016). Adipose tissue dysfunction is described by hypertrophic adipocytes that display an increase in adipokines secretion (Longo *et al*., 2019; Fuster *et al*, 2016). The secretion of adipokines, largely coordinated by reduced adiponectin and improved resistin, leptin, interleukins (IL-6, IL-8, IL-1β), tumor necrosis factor-α (TNF-α), monocyte chemo-attractant protein (MCP-1), attracts and triggers immune cells (Paz-filho *et al.*, 2012).

Obesity leads to recruitment of macrophages and a polarization toward their M1 pro-inflammatory phenotype (Lacy *et al.*, 2016). M1 macrophages synchronize an important part of obesity inflammation and discharge pro-inflammatory cytokines such as IL-6 and MCP-1. M2 macrophages alternatively secrete anti-inflammatory cytokines like IL-10 (Lacy *et al.*, 2016). Immune cells such as lymphocytes, mast cells, dendritic cells, eosinophils, killer cells, and foam cells have been found to infiltrate obese WAT at a high rate in obese conditions contributing to the systemic inflammation (Chimenti *et al*., 2015).

Increased circulating levels of pro-inflammatory cytokines and fatty acids (FAs), affect insulin function and signaling in metabolic tissues, producing systemic inflammation and insulin resistance (Yao *et al*,. 2014). As a result, there is an impaired glucose disposal in muscle and better lipolysis in adipose. In turn hyperinsulinemia, hyperglycemia, hyperlipidemia seem to contribute to the progression of obesity linked comorbidities, comprising type 2 diabetes and cardiovascular diseases (Yao *et al.*, 2014).

Other adipose cell fraction comprises of stromal and epithelial cells, pre-adipocytes, fibroblasts as well as the innate immune system cells principally macrophages, adaptive immune system cells primarily T cells, natural killer (NK) cells, (Henegar *et al.*, 2008) and mast cells (Liu *et al.*, 2009). The phenotype of the macrophages change depending on the tissue microenvironment. There are

nearly about 10 per 100 adipocytes macrophages representing roughly 15% of stromal cells in normal adipose tissue of humans (Aron-Wisnewsky *et al*., 2009). They are mostly located in the parenchyma fat between the adipocytes and blood vessels. T cells are less common and are between 6 and 10% whereas B cells are not detectable (Aron-Wisnewsky *et al.*, 2009; Duffaut *et al.*, 2009).

2.1.1 Histology of adipose tissue

The white adipose tissue and the brown adipose tissue have distinct histological and cellular properties (Berry *et al.*, 2013). White adipose tissue cells contain single large unilocular lipid droplets and few mitochondria whereas brown adipocytes mainly contain numerous multilocular lipid droplets including enormous amount of mitochondria (Contreras *et al.*, 2015; Villarroya *et al*., 2013). Cells of white and brown adipose tissues alter their structure and function due to their heterogeneity and plasticity when energy demands are altered under certain physiological and pharmacological conditions as in high-fat diet intake (Kotzbeck *et al*., 2018).

The cells of white adipose tissues are large round cells as shown in figure 1, whose diameter vary in different proportions from about 10μm to 120μm in diameter (McLaughlin *et al*., 2007). The nuclei are peripheral to the cell and appears “empty” due to histological processing. The cells in white adipose tissue are structured into lobules by connective tissue septa which consist of collagen fibers, nerve endings, and blood and lymph capillaries (Estève *et al.*, 2019). Reticular fibers constitute the extracellular matrix of white adipose tissue and contains non-residential cells such as inflammatory cells. It becomes polyhedral when crowded into adipose tissue and continuously store fat in the form of a single droplet bounded by no membrane (Le Lay *et al*., 2015). The

cytoplasm and the nucleus are displaced peripherally against the plasma membrane, giving the cells a signet ring profile when viewed by light microscope (Bastard & Fève, 2012).

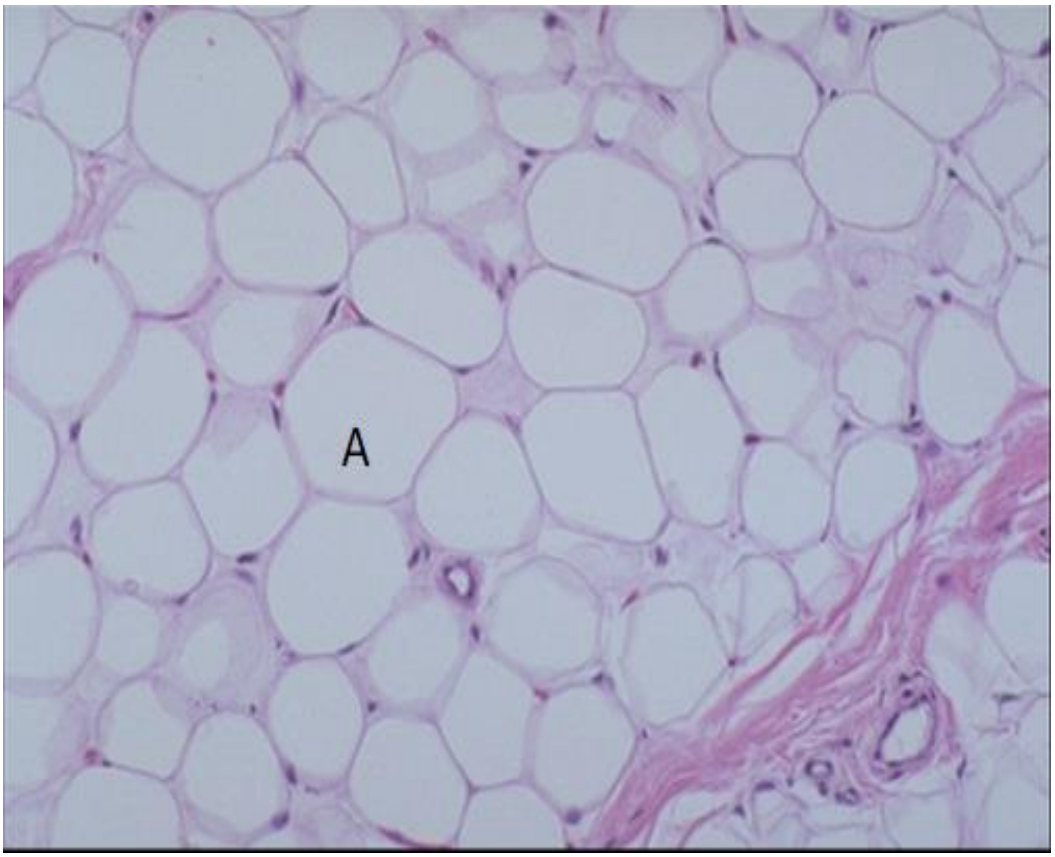


Figure 1: Photomicrograph showing microscopic anatomy of white adipose tissue. Adipocyte labeled (A) are located in the connective tissue.
https://webpath.med.utah.edu/histhtml/normal/norm141.html. Assessed on 07/09/2020.

2.1.2 Functions of adipose tissues

Adipose tissue plays a vital role in energy metabolism through its capacity to store energy in the fed state, and to discharge it in the starving state in the form of triglyceride and free fatty acids (Parker, 2018). This basic function of adipose tissue is of critical significance to life and its essential mechanisms are preserved from yeast to primates (Birsoy *et al.*, 2013).

In addition to energy storage, adipose tissue has other vital functions. It influences systemic energy homeostasis facilitated in part through the endocrine system. Hormones discharged from adipose tissue are called adipokines with leptin being the first adipokine to be described which acts on the central nervous system to suppress appetite and stimulates energy expenditure (Caruso *et al.*, 2010). Adiponectin also acts centrally to influence energy expenditure, as well as improving systemic insulin sensitivity, and has paracrine effects to increase adipocyte mass (Czech, 2020).

2.2 Gross anatomy of the liver

The liver is the second largest organ in the human body. It is situated in the upper right quadrant of the abdominal cavity, weighing approximately 1500g and accounts for 2% body mass of an adult human being (Riestra-Candelaria *et al.*, 2016). It serves as a nutrient storage organ and produces bile for the digestion of fat. It is reddish brown in color which is dependent on the amount of venous blood volume it contains and the unique blood supply and features of the liver makes it susceptible to possible fatal injuries when it splits open (Ellis, 2011).

The human liver is partitioned into lobes comprising the right, left, caudate and quadrate lobes with surface peritoneal and ligamentous attachment (Vdoviaková *et al.*, 2016). Superiorly, it has falciform ligament with ligamentum venosum inferiorly located, which mark the partition between right and left lobes (Ellis, 2011).

2.2.1 Embryology of the liver

Human liver development commences during the third to fourth week of gestation (Giancotti et al., 2019). The endoderm germ layer is established during gastrulation and forms a primitive gut tube that is subdivided into foregut, midgut and hindgut regions. The embryonic liver originates from the ventral foregut endoderm (Giancotti *et al.*, 2019; Wells & Melton, 2000). The first morphological sign of the embryonic liver is the formation of the hepatic diverticulum, an out-

pocket of thickened ventral foregut epithelium adjacent to the developing heart (Zorn, 2008). The anterior portion of the diverticulum gives rise to the liver and intrahepatic biliary tree, while the posterior portion forms the gall bladder and extrahepatic bile duct (Higashiyama et al., 2018). The hepatic endoderm cells, known as hepatoblasts delaminate from the epithelium and invade the adjacent septum transversum mesenchyme to form the liver bud (Zorn, 2008). The septum transversum mesenchyme contributes fibroblast and stellate cells of the liver. Several transcription factors, as well as signals from endothelial cells are required for the development of the liver (Higashiyama et al., 2018). The liver undergoes a period of accelerated growth as it is vascularized and colonized by hemapoietic cells to become the major fetal hematopoietic organ. The majority of hepatoblasts in the parenchyma differentiate into hepatocytes (Soares-da-Silva *et al*., 2020). The maturation of functional hepatocytes and the formation of a biliary network connected to the extrahepatic bile duct are gradual. This process continues until after birth to generate the characteristic tissue architecture of the liver (Soares-da-Silva et al., 2020).

2.2.2 Histology of the liver

The liver is organized into lobule and acinus as the functional operational units (Krishna, 2013). Histologically, hexagonal lobules are oriented around terminal hepatic venules or central veins as shown in figure 2 (Mak & Png, 2020; Greaves, 2012). Portal triads (or portal tracts) are at the edges of the lobule, containing a branch of the portal vein, a hepatic arteriole, and a bile duct as shown in figure 2. Blood enters the portal tract via the portal vein and hepatic artery (Goldin, 2017). Blood enters the sinusoids and penetrates along the cords of parenchymal cells (hepatocytes). Finally the blood runs into the hepatic venules, and leaves the liver through the hepatic vein (McCuskey, 2008).

Sinusoids are bigger and more irregular than normal capillaries as shown in figure 2. Kupffer cells, endothelial cells, and stellate cells are associated with the sinusoids (Demetris *et al.*, 2016). The liver, in addition has a substantial amount of lymphocytes, particularly natural killer (NK) and natural killer T (NKT) cells (Gao *et al.*, 2015). Sinusoids are lined by thin, discontinuous endothelial cells with numerous fenestrae (or pores) that permit molecules smaller than 250 kDa to cross the interstitial space (known as the space of Disse) between the endothelium and hepatocytes (Jacobs *et al*., 2010). Sinusoidal endothelial cells are separated from the hepatocytes by a basement membrane-like matrix, which is not as electron-dense as a regular basement membrane (Ni *et al*., 2017; Wambaugh & Shah, 2010).

However, this sub-endothelial extracellular matrix is significant for the normal function of all resident liver cells (Friedman, 2000). Endothelial cells are important in the scavenging of lipoproteins via the Apo-E receptor and of denatured proteins and advanced glycation end-products by the scavenger receptor. Kupffer cells are the resident macrophages of the liver and constitute approximately 80% of the fixed macrophages in the body (Kakinuma *et al*., 2017). Kupffer cells are sited within the lumen of the sinusoid. The crucial function of Kupffer cells is to ingest and destroy particulate matter. Hepatic stellate cells or Ito cells or by the more descriptive terms of fat-storing cells are located between endothelial cells and hepatocytes (Senoo, 2004).

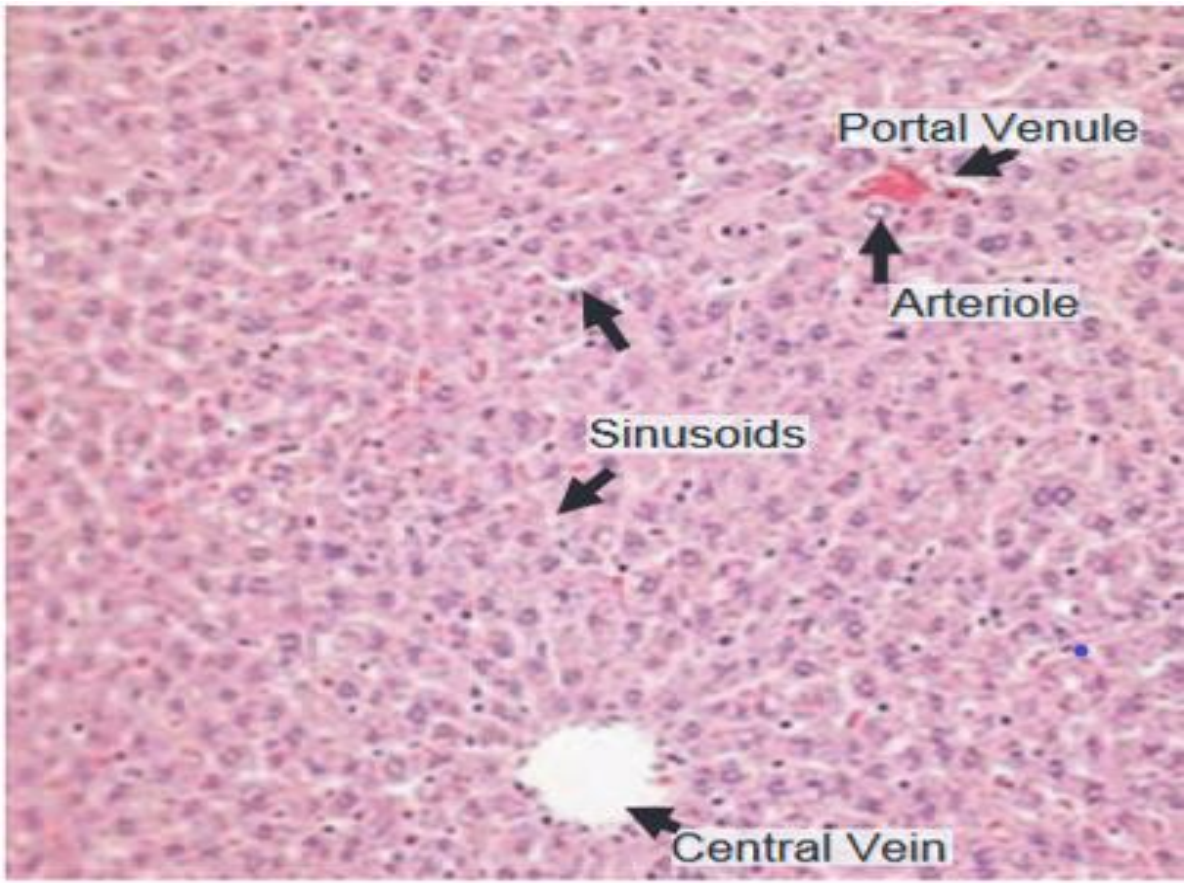


Figure 2: photomicrograph showing microscopic Anatomy of the liver (Wambaugh & Shah, 2010)

2.2.3 Anatomy of the rat liver

The rat's liver has a mass of approximately 5% of the total body weight and is multi lobulated compared to other mammals (Vdoviaková *et al*, 2016). The rat liver fundamentally has three surfaces: inferior, superior, and posterior. The inferior surface is separated from the superior surface by a sharp margin which is different from that of the human liver (Ney *et al*, 2017). The rats liver lobes have uniform surfaces as they lie flat against each other. The posterior caudate lobe is however separated by the stomach from the remaining lobes. The superior (parietal) surface consist of a part of the left lateral and medial lobes and is entirely covered by the peritoneum, except along the line of attachment of the falciform ligament (Ney *et al*, 2017). The line of

attachment of the falciform ligament divides the liver into right and left lobes. The right lobe of the human liver is much larger than the left whereas the left and right liver lobes of the rat have the same volume. The inferior (visceral) surface is irregular, concave and does not have the fossae in the shape of the letter 'H' as in humans (Ney *et al*, 2017). The hepatic artery, the portal vein, and nerves, the hepatic duct and lymphatic enter through the portal or transverse fissure. Liver impressions (duodenal, colic, renal, and suprarenal) are not as pronounce as in the liver of humans (Vdoviaková *et al.*, 2016). The posterior surface is not covered by the peritoneum over some part of its extent, and is directly in contact with the diaphragm. The inferior vena cava is completely intrahepatic (Ney *et al*., 2017).

2.2.4 Functions of the Liver

The functions of the liver include synthesizing glucose from amino acid and other organic molecules , the breakdown of glycogen into glucose, and the formation of glycogen from glucose and amino acid synthesis (Lehot *et al.*, 1992). The liver controls glucose levels in the bloodstream (glucose homeostasis) and plays critical roles in the metabolism of lipids as well as the synthesis of cholesterol and the production of triglycerides or fat (Alves-Bezerra & Cohen, 2018).

The liver is involved in the breakdown of insulin, hemoglobin, ammonia, toxic substances, and most medicinal products. The liver secretes bile, converts several proteins for storage, produces certain proteins for immunological effects and regulating blood pressure (Lehot *et al*., 1992). In all the liver performs critical roles including digestion, detoxification, homeostasis and fluid and electrolyte balance.

2.3 High-fat diet metabolism, obesity and related complications

2.3.1 Composition and groups of fat

Fats, carbohydrates and proteins are the three macronutrients in diets. Fats are consumed in the form of triglycerides, a molecule made up of three fatty acids attached to a glycerol backbone (Lowery, 2004). Fats are categorized as short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA) and long-chain fatty acids (LCFA) as indicated by Nagy & Tiuca (2017). Most of the fats consumed are MCFA and LCFA which are imbibed into the bloodstream and released into the body cells for energy (Nagy & Tiuca, 2017). Fat contains 9 calories of energy per gram whilst proteins and carbohydrates offer 4 calories per gram. Fatty acids are grouped into monounsaturated fatty acids, polyunsaturated fatty acids and saturated fatty acids based on the number of double bonds found in their structure (Forouhi *et al.*, 2018).

There is only one double bond in monounsaturated fats whereas polyunsaturated fats are characterized by two or more double bonds (Forouhi *et al.*, 2018). Both monounsaturated and polyunsaturated fats are connected with several health benefits including reduced risk of obesity, heart diseases and diabetes (Forouhi *et al.*, 2018). However, saturated fatty acids have no double bonds in their chain and intake have been associated to various health complications such as raising levels of low density lipoprotein (LDL) cholesterol in some humans and animals (Forouhi *et al.*, 2018). In their dietary guidelines, the World Health Organization and the Dietary Reference Intakes recommend that a total fat intake must be between 20 and 35% of total calories (American Heart Association, 2015).

2.3.2 How is fat metabolized?

Dietary fat is a critical source of energy supplying about 9 calories per gram of energy, about twice the amount supplied by protein or carbohydrate (Street, 2002). Metabolism of lipids commences

in the small intestine where consumed fats or triglycerides are broken down into fatty acids (small chain) by the action of bile salts and pancreatic lipase (Goodman, 2010). Cholecystokinin (CCK) which is a digestive hormone, is discharged when ingested fat reaches the small intestine by the intestinal cells (Liddle, 1995). Cholecystokinin activates the gall bladder and pancreas to respectively release bile salt and pancreatic lipase into the intestine (Le *et al*., 2019). The bile salt and the pancreatic lipase together breakdown triglycerides into FFA and they are carried into the intestinal membranes where they come together to form again triglyceride molecules (Cox & Garcia-palmieri, 1990). These triglyceride molecules are packaged along with cholesterol molecules within the intestinal cells in phospholipid vesicles called chylomicrons (Cox & Garcia-palmieri, 1990). The chylomicrons by exocytosis leave the enterocytes into the lymphatic system and then transport them to the circulatory system where they either move into the liver or are stored in the adipocytes (Xiao *et al.*, 2019).

High-fat diet increases the level of chylomicrons in the intestine and enter circulation leading to the production of FFA which are absorbed by the liver (Pessayre *et al.*, 2001). These free fatty acids in the liver either move into mitochondria for β-oxidation or be esterified into triglycerides. The triglycerides pile up in the hepatocytes as small droplets or produce very low- density lipoprotein, which is then converted into low-density lipoprotein (Pessayre *et al*., 2001).

2.3.3 High-fat diet intake and obesity in humans and animals

Regular consumption of high-fat diet results in excess body fat in mice, rats and other animals as shown by (Preguiça *et al*., 2020). High-fat diet comprising 30% or more of total energy from fats lead to weight gain and subsequently obesity as a result of high energy consumption and effective energy storage (Elimam & Ramadan, 2018; Hariri & Thibault, 2014). Several reports reveal that animals receiving high-fat diet take less amount and prolonged food intake of lipid-rich diet

induces weight gain in susceptible rats (Timmers *et al.*, 2010; Buettner, 2007). It has been revealed that rats ingesting high-fat diets maintain metabolic homeostasis for four to six weeks before they start to gain weight and develop obesity and its related complications including insulin resistance, cardiovascular diseases and type 2 diabetes (Buettner, 2007). A study conducted by Xie *et al.*, (2008) demonstrates that animals receiving high-fat diet had a greater quantity of visceral fat and accumulation of fatty acid in the liver as well as reduced glucose tolerance and insulin resistance.

2.3.4 Obesity and adipose tissue dysfunction

Studies conducted on animals have shown that high-fat diet is an essential player in the etiology of obesity (Preguiça et al., 2020). When adipose tissue storage capacity for free fatty acids is exceeded due to nutrient overload, it expands to accommodate the excessive caloric intake and this changes the structure and cellular composition (van Herpen & Schrauwen-Hinderling, 2008). This shift in morphology promotes obesity and dysfunction (Fuster *et al*., 2016). This obesity-associated remodeling of the adipose tissue as shown in figure 3 generates systemic pro-inflammatory state mediated by the production of adipokines such as TNF-α, IL-1β and IL-6 which affect insulin sensitivity and ultimately contribute to the development of cardiovascular diseases and type 2 diabetes (Fuster *et al.*, 2016; Guillemot-Legris *et al*., 2016; Gao *et al*., 2015; Unger & Scherer, 2010).

Obesity is known to be the principal risk factor for chronic diseases such insulin resistance, cardiovascular diseases, type 2 diabetes and some types of cancer (El Mouzan *et al.*, 2010). Obesity is rapidly increasing with 400 million obese and 1.6 billion overweight of adults living with the disorder globally (El Mouzan *et al.*, 2010). This increase in obesity around the world in such a short period can be linked to increased dietary fat intake, although genetics play critical role in body weight, body size and response to feeding in animals (Guerre-Millo, 2013) and humans

(Ichihara & Yamada, 2008). Studies have revealed that, as the average amount of fat in the diet increases, the incidence of obesity also increases (Saris *et al*., 2000).

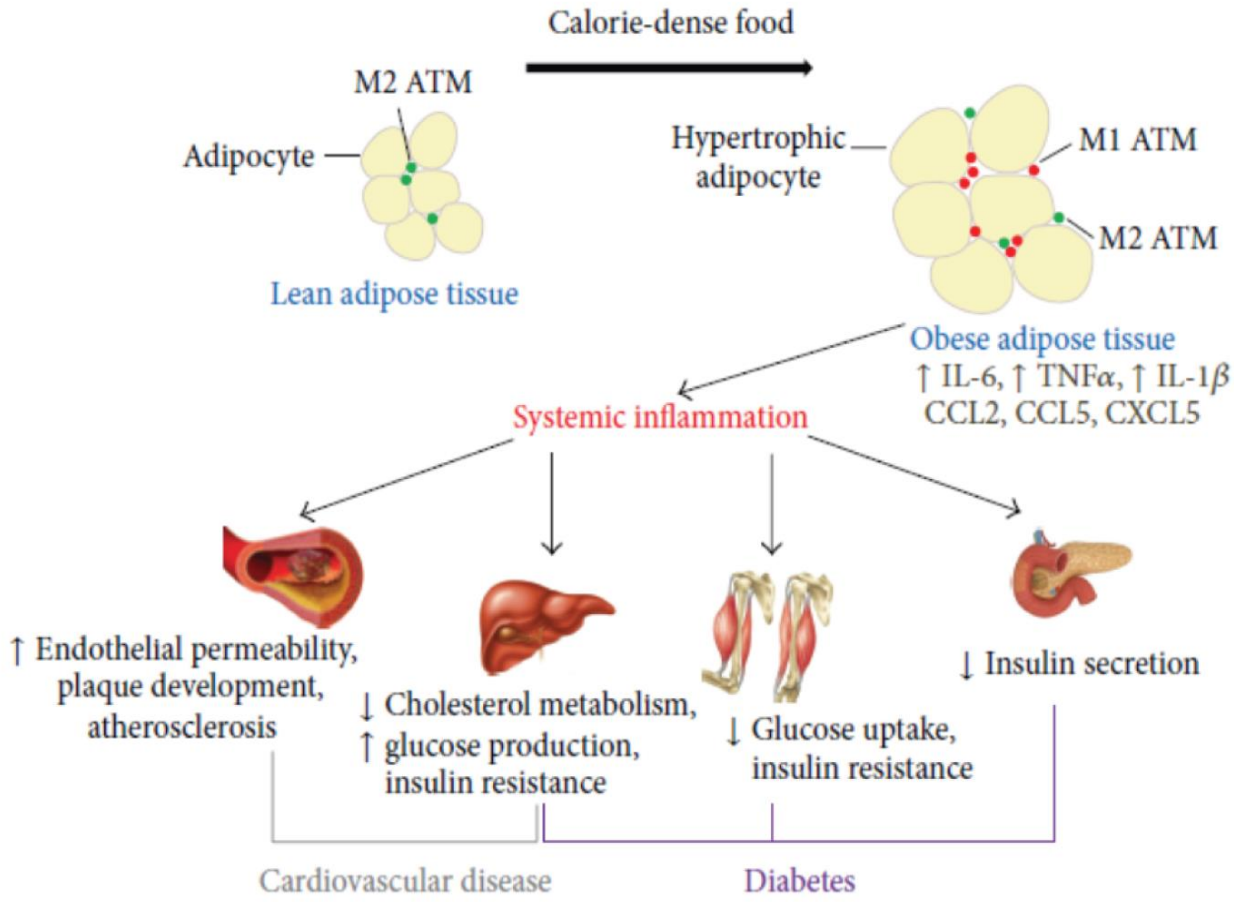


Figure 3: Diagram showing mechanism by which calorie dense diets (high-fat diets) results in obesity and its related complications (Yao *et al*., 2014).

Frequent intake of calorie dense (high-fat) diet leads to obesity which is concomitant with adiposity including hypertrophy of adipose tissue and influx of pro-inflammatory monocyte that mature and stimulate M1 ATM as shown in fig. 3 (van der Heijden et al., 2015; Yao et al., 2014). Ordinary adipose tissue is populous with otherwise stimulated M2 ATM. The various pro-inflammatory cytokines produced include IL-6, IL-1β and TNFα and various chemokine such as CXCL5, CCL2, and CCL5 (Turner *et al*, 2014). Immune cells and adipocytes initiate adipose

tissue inflammation and subsequently systemic inflammation with prolonged high-fat diet intake as shown in figure 3. This systemic inflammation affects endothelial permeability (cardiovascular diseases), insulin action (Duan *et al*., 2018) and signaling in metabolic tissues (liver and skeletal muscles) producing insulin resistance and impair glucose function. The systemic inflammation stimulate hyperinsulinemia, hyperglycemia, and hyperlipidemia contributing to cardiovascular diseases, type 2 diabetes and other cancers (Ramesh *et al.,* 2013; Yao *et al*., 2014).

2.4 Effects of high-fat diet on the adipose tissue function

Diets high in fat are recognized to lead to a positive fat balance and accordingly to adipose mass accumulation (Fonseca *et al.,* 2018). Reports suggest that adipocyte sizes in epididymal adipose tissues increased to a greater extent in individuals exposed to both high-fat diet compared with those on low-fat (Poret *et al.*, 2018). Choe *et al.*, (2016), suggested that the administration of high-fat diets produced changes in the morphology and size of adipocytes and pancreatic islets, producing chronic insulin and leptin release.

Further findings suggest that the size of adipocytes is a major regulator of their endocrine function with hypertrophic adipocytes secreting high amount of TNF-α and free fatty acids (Chen & Farese, 2002). Hypertrophic adipose morphology is positively correlated with insulin resistance, diabetes and cardiovascular diseases (Tandon *et al.*, 2018) . Compared to rats on regular chow, high-fat diet fed rats progressively gained more fat mass and subsequently displayed accelerated body weight gain, which was accompanied with adipocyte hypertrophy and up-regulated manifestation of adipose inflammatory chemokine and cytokines such as monocyte chemo-attractant protein (Mcp-1) and TNF-α (Gao *et al*., 2015).

Overload of fat in white adipose tissue consequently leads to ectopic fat deposition in other tissues such as brown adipose tissue, giving rise to whitening of brown adipose tissue (Gao *et al.*, 2015).

Particularly, adipose tissue chronic inflammation and ectopic lipid deposition in the liver and the brown fat are accompanied by glucose intolerance and insulin resistance which is connected with hyper insulinemia and pancreatic islet hypertrophy (Ghaben & Scherer, 2019).

2.5 Effects of High-fat diet on the liver

The adult human liver has a low lipid content (below 5% of fat by wet weight) and when this capacity is surpassed, it is termed as fatty liver or liver steatosis (Friedman *et al.*, 2018). Alcohol abuse is the main well-known cause of a fatty liver and also genetic predisposition increases the risk of developing the diseases (Farooq & Bataller, 2016). Mostly, overweight and obesity are strong risk factors for non-alcoholic fatty liver disease (NAFLD) and as the incidence of obesity is reaching epidemic proportions, a significant part of the population is likely to be affected by liver steatosis (Farooq & Bataller, 2016). It has been reported from the United States (US) and Japan that up to one third of the general population may have a fatty liver (Younossi, 2019).

Studies in animals and humans have shown that diets containing high amount of fat rapidly enhance hepatic steatosis and lipid accumulation in the liver while low-fat diets decrease liver fat content (Jensen *et al*., 2018). This suggests that high-fat diet has detrimental effect on the health of an individual. According to Buettner, (2007), high-fat diet may trigger hepatic steatosis, hepatic insulin resistance, hyperglycemia in most animals as observed in obese humans and reveal that the mechanism by which the diet stimulates fat deposition as well as non-alcoholic steatohepatitis in rats may be the activation of hepatic inflammation (Buettner, 2007).

Fatty liver diseases are characterized predominantly by large droplet steatosis (macro vesicular steatosis), or mixed large and small droplets steatosis (Tandra *et al.*, 2011). Small droplets (micro vesicular) that do not fill the entire hepatocytes are usually included in the macro vesicular category (Tandra *et al.*, 2011). Several primary liver diseases, such as hepatitis C, Wilson diseases,

hepatocellular adenoma and carcinoma, and certain drugs and toxins, such as steroids and alcohols commonly show these signs of steatosis (Guy & Peters, 2013). Exposure to high-fat diet leaves droplets in the hepatocytes since the liver is the organ that metabolizes and processes lipids (Osna *et al*., 2017).

2.6 Clinical presentation of high-fat diet on the liver

Non-alcoholic fatty liver diseases (NAFLD) encompasses a range of liver conditions affecting individuals who do not take alcohol, and other established liver diseases (Abd El-Kader & El-Den Ashmawy, 2015). NAFLD is characterized by hepatic accumulation of fat, more than 5% of liver weight and can exist as pure steatosis, steatosis with mild lobular inflammation, an aggressive form as non-alcoholic steato-hepatitis (NASH) and may progress to advanced scaring (cirrhosis) and hepatocellular carcinoma as well as liver failure (Lindenmeyer & McCullough, 2018).

Non-alcoholic fatty liver disease is caused by the accumulation of fatty acid (triglycerides) in the liver and is linked to overweight or obesity, insulin resistance, hyperglycemia, pre-diabetes or type 2 diabetes, and hyperlipidemia particularly triglyceride (Hazlehurst *et al.*, 2016). These appear to promote the deposit of excess fat in the liver and act as a toxin causing injury to the liver and alter the morphology of liver cells (Liu *et al*., 2010). Individuals with NAFLD have higher mortality from both liver and non-liver-associated causes and have increased risk for health related problems including cardiovascular diseases (Friedman *et al.*, 2018), type 2 diabetes and chronic kidney disease (Stefan *et al*., 2019).

The disease (NAFLD) typically presents between the ages of 35-50 years with slight increase in aminotransferase, though the disease may also occur in childhood (Harrison & Neuschwander-Tetri, 2004). Patients with the disease may experience clinical conditions comprising the metabolic

syndrome such as obesity, dyslipidemia, diabetes, and hypertension, whereas others may be asymptomatic with few presented with fatigue or a vague, nondescript pain or discomfort in the upper right quadrant (Chitturi *et al*., 2002). There is also elevated levels of ferritin which is a marker of insulin resistance in about 40-50% of patients (Chitturi *et al*., 2002; Angulo *et al.*, 1999).

2.7 High-fat diet generate oxidative stress

Consumption of saturated fatty acid food which is generally found in animal products (cream, red meat, butter and whole milk dairy products) and some plant products (coconut oil, palm oil and palm kernel oil) is associated with impaired glutathione metabolism and enhanced ROS production, resulting in the development of NAFLD (Mozaffarian *et al.*, 2010) and oxidative stress (Flores, 2019). When the energy supply begins to exceed the storage capacity of adipocytes , inflammation and oxidative stress occur resulting in hypertrophy of adipocytes (Klöting & Blüher, 2014). This hypertrophy leads to a higher discharge of adipokines as pro-inflammatory cytokines such as interleukin- 1 (IL-1), interleukin- 6 (IL-6) and tumor-necrosis factor alpha (TNF-α), leading to low-grade chronic inflammation which emanates from adipose tissue and ultimately extends in circulation to other organs (Cotillard *et al.*, 2014). One of the leading effects of inflammation is insulin resistance which is as a result of the prevention of phosphorylation of insulin receptors by TNF-α, interfering in their cascade action and preventing their functioning (Lauterbach & Wunderlich, 2017)

2.8 Effect of high-fat diet on inflammation

The mechanism of inflammatory response upon high-fat intake begins with activation of Toll-like receptor (TLR) signaling pathway resulting in increased penetrability to endotoxins such as lipopolysaccharide (LPS), promoting their presence in circulation (Bleau *et al.*, 2015; Ding *et al*., 2010; Kim *et al*., 2012). High levels of LPS and FFA trigger the production of pro-inflammatory

cytokines including IL-1β, IL-6 and TNF-α in the gut (Antonioli *et al.*, 2019; Konrad & Wueest, 2014).

The elevated levels of LPS, FFAs, and pro-inflammatory cytokines in the systemic circulation result in systemic low-grade inflammation (Konrad & Wueest, 2014; Tsukumo et al., 2015). The expression of TLRs in circulating macrophages may be upregulated by increased plasma FFAs and LPS facilitating the activation of pro-inflammatory macrophages (M1 phenotype) and subsequent production of pro-inflammatory cytokines (Bleau *et al.*, 2015; Ding *et al.*, 2010) as shown in figure 4.

These factors stimulate inflammatory pathways in the brain before the inception of obesity (Zhang *et al.*, 2008). Increased levels of FFAs and cytokines initiate inhibitor of kappa B (IKB) kinase β/ nuclear factor of kappa B (NF-kß) signaling directly or indirectly through stimulating TLR site at cellular surface or through stimulating various cellular stresses involving oxidative stress and endoplasmic reticulum stress in the hypothalamus (Cai & Liu, 2011). Stimulated IKKβ and Nf-KB signaling shuts central leptin and insulin sensitivity and triggers gene expression of inflammatory response (Zhang *et al*., 2008; Woods *et al*., 2004). Elevated inflammatory macrophages (M1) in circulation reach adipose, liver and muscular tissues, pancreatic islets, and blood vessels resulting in peripheral inflammation (Lumeng & Saltiel, 2011) as shown in figure 4. Accumulation of clusters of differentiation ($CD8^{+}$) T-cells in the AT exacerbates macrophage (M1) recruitment in the AT (Lumeng & Saltiel, 2011).

The intake of HFD puts stress on the adipose tissue failing to store the excess lipids and therefore depositing them into tissues including the liver, pancreas, skeletal muscles, and blood vessels (Lumeng & Saltiel, 2011) as shown in figure 4. Lipid accumulation in the tissues contribute to

extra expression of pro-inflammatory mediators and the more recruitment of MI macrophages, exacerbating systemic inflammation (Caesar *et al*., 2015; Lee & Lee, 2014). The liver is also exposed to increased levels of different mediators such as pro-inflammatory cytokines, LPS, and FFAs released by the gastrointestinal tract (Konrad & Wueest, 2014). These mediators lead to accumulation of Natural killer T (NKT) cells and stimulation of Kupffer cells in the liver, contributing to hepatic and systematic inflammation (Bhattacharjee *et al*., 2017).

High-fat diets elicit a complex system of signals connecting different organs to act in synergy to stimulate a low-grade systemic inflammation (van der Heijden et al., 2015) as shown in figure 4. HFD- associated inflammation results in failure of adipocytes to efficiently remove circulating FFAs which is pivotal to disease development and the progression of complications including T2DM, CVD, liver disorder, atherosclerosis, and certain types of cancers (Duan *et al*., 2018).

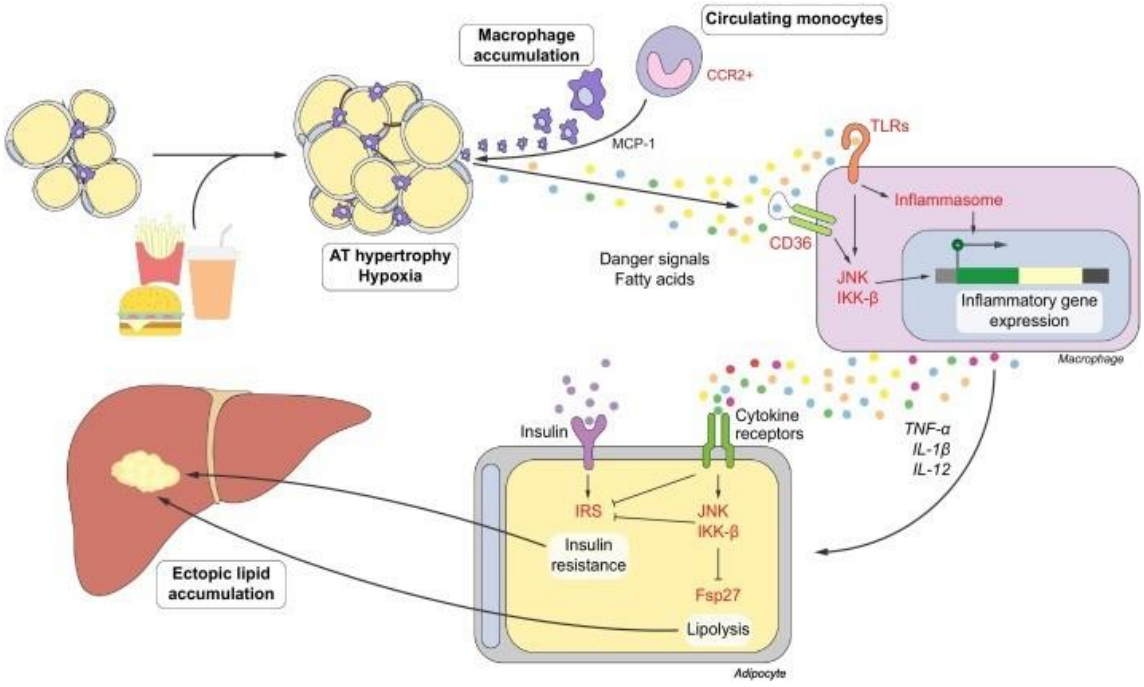


Figure 4: Diagrammatic representation of high-fat diet stimulating metabolic inflammation in an organism. Obesity leads to adipose tissue dysfunction, triggering the release of pro-inflammatory

adipokines (group of chemicals released by different immune cells) that can directly act on cardiovascular tissues to promote disease (Fuster *et al*., 2016).

The biological functions of these adipokines overlap and can be injurious in the condition of high-fat diet ingestion over a long term period (Duan *et al*., 2018). TNF-α is a pro-inflammatory cytokine primarily produced by macrophages infiltrated in AT, Kupffer cells and hepatocytes that are characterized by various biological effects involving metabolic, proliferative but also necrotic (Nagarajan *et al*., 2012). Evidence suggests that TNF-α is directly toxic to liver cells and induce the production of other cytokines, attracting leucocytes to release ROS and toxic enzymes (Nagarajan *et al*., 2012). It also has a role in the progression of NAFLD conditions involving liver steatosis, apoptosis, necrosis and fibrosis as well as insulin resistance (de Andrade *et al*., 2015; Li *et al*., 2015).

IL-1 cytokines are secreted by macrophages and Kupffer cells and are grouped into pro-inflammatory comprising IL-1β and IL-18 and anti-inflammatory constituting IL-1Ra. Studies have proven that IL-1β and IL-1α function in the transformation of steatosis to steatohepatitis and liver fibrosis (Tan *et al*., 2016).

2.9 Role of high-fat diet on Insulin sensitivity and glucose tolerance

Obesity leads to elevated insulin levels in plasma and metabolic resistance to the effect of insulin (Czech & Building, 2018; Kahn & Flier, 2000). In the absence of obesity, high-fat consumption contributes to impairing glucose tolerance and insensitivity to the blood glucose-lowing effect of insulin (Czech & Building, 2018). It has been shown both in animals and humans that elevated plasma lipid levels by high-fat diet results in intracellular lipid accumulation in skeletal and liver tissues (Czech & Building, 2018). This ectopic lipid deposition due to lipid overload is considered to be a major factor of insulin resistance (Ormazabal et al., 2018). Evidence from epidemiological

studies show that, insulin sensitivity is impaired when the total energy intake is about 35-40% (Parillo & Riccardi, 2004). Consumption of SFA in humans correlate positively with glucose and insulin levels in individuals (Imamura *et al*., 2016). The ectopic accumulation of fat may increase secretion of insulin but reduce its ability to drive glucose uptake (Woods *et al*., 2018). In both female C57BL/6J mice and Sprague-Dawley male rats fed on high-fat diet with diverse proportions of energy as fat, showed a direct association between the percentages of fats in diets and glucose intolerance (Gallou-kabani et al., 2007; Ahre, 2004).

Abdul-Ghani & Defronzo, (2010) and Parillo & Riccardi, (2004) suggested that abnormalities such as reduction in insulin receptors, metabolism and glucose transport as well as decline in liver and synthase activity in muscle glycogen and storage of glycogen from glucose conversion, developed when the fat intake is 40% or high of total energy. Studies also show that endoplasmic reticulum (ER) stress in the AT lead to cytokines secretion which in turn decrease the receptiveness of the cells to insulin (Peterson *et al.*, 2017). High concentration of fatty acids in diets affect the configuration of the cell membranes which influences the affinity of receptors for insulin and its action on cells (Perona, 2017). Reports from some studies support the notion that insulin secretion and sensitivity are improved as the degree of unsaturation of fatty acids increases and thus feeding on diets rich in SFA leads to more insulin resistance than in MUFA and PUFA (Iggman & Risérus, 2017; Imamura *et al.*, 2016; Coelho *et al*., 2011).

2.10 Animal models of high-fat diet

In rats fed with high-fat diets, a linear growth in body fat with increasing body weight has been revealed (Soliman et al., 2019). Conversely, results of the study by Schölmerich & Bollheimer, (2002) indicated that assessing body fat is a more sensitive benchmark for assessing obesity in animals since rats fed a high-fat diet (40% of energy) for 10 weeks showed a 10% increase in total

body weight but a 35-40% rise in total body fat compared to animals fed a low–fat diet. Three types of diet were offered to Sprague-Dawley rats by Shiraev, *et al*, (2009), control (14% lipids), ad libitum HF (35% lipids) and HF isocaloric to the control for 11 weeks and observed increased body fat, glucose intolerance and elevation of serum insulin levels in the HF diet groups. The conclusion was that the presence of large amounts of fat in the diet even with low calorie content can result in increased adiposity and hyperlipidemia leading to insulin resistance on long-term basis (Shiraev, *et al.*, 2009).

Dietary obesity of animal models are categorized as susceptible and resistant based on their body fat or weight gain (Tulipano *et al*., 2004). Sprague- Dawley rats exposed to high-fat diet were classified based on their final body weight, with rats in the highest percentage denoted as obesity prone and those in the lowest percentage consigned as resistant as indicated by Giles *et al*., (2016). Studies on humans have shown important positive relationship concerning the amount of dietary energy from fat and the percentage of the individuals who are overweight and between the level of dietary fat and the gain in body weight as well as between the decrease in the fat in the diet and loss of weight (Raatz *et al*., 2017). These relations have been confirmed in animal studies as reported by (Ghibaudi *et al.*, 2015). This indicates the relationship in humans or animal models of more dietary fat resulting in greater obesity demonstrates that the fat content of the diet is critical factor in energy balance.

The composition of fatty acids in the diet plays an essential role in regulating body weight and cellularity of the AT (Moussavi *et al*., 2008). Saturated fatty acids (SFA) encourage obesity in human subjects and this notion has been supported in studies of animal subjects by presenting increased accumulation of body fat (Coelho *et al.*, 2011) and higher body weight on ingesting moderate diet or high diet containing SFA (Aller *et al*., 2011; Bray & Popkin, 1998). Ellis *et al*.,

(2002) conducted a study on Sprague-Dawley rats matching diets rich in low and high SFAs for two months revealed increased number of fat cell in animals fed with coconut oil and greater fat cell size in the rats fed maize oil. These results show that adverse forms of obesity developed from consuming a diet high in SFA since hypertrophy of adipocytes is a prerequisite for hyperplasia (Ellis *et al*., 2002). Rats exposed to a high saturated fat diet (58% total calories) for 7 weeks put on greater adiposity when compared to omega 3 group and 10% low fat-diet of the control group (Coelho *et al*., 2011).

Saturated fatty acid (SFA) induces obesity due to the fact that SFA is poorly utilized for energy and continues to be acylated into triglyceride and stored in AT though polyunsaturated fatty acid (PUFA) and monounsaturated fatty acid (MUFA) are easily utilized for energy and so stored less (Albracht-Schulte *et al.*, 2018).

A study conducted by Okere *et al.* (2006) confirmed that feeding of high-fat in adult Wistar rats for 8 weeks showed a greater intra-thoracic fat mass in animals fed on diet containing rich SFA whereas those fed on diet containing PUFA showed greater intra-abdominal and epididymal fat mass.

2.11 Natural cocoa and its health benefit in HFD fed animals

2.11.1 Histological background and benefits of natural cocoa

Since 600 BC, Cocoa which is a derivative of *Theobroma cacao* plant has been ingested by the ancient Mayans and Aztecs (Hurst *et al.*, 2002). Cocoa, one of the world's most popular food products has rich history encompassing several cultures carrying substantial social and economic implications to masses of individuals in the globe (Araujo *et al.*, 2016). The Mayans were the first to consume cocoa and gave its ancient name "kakawa" which translates into "Food of the Gods"

concerning its multiple health benefits (Rusconi & Conti, 2010). There are three varieties of cocoa beans used to make cocoa products. The less bitter and more aromatic, highly prized and mostly used by Mayas is the Criollo cocoa tree from which about 5-10% of world's chocolate are made (Selmi *et al.*, 2008). Another group that is hardier than *Criollo* trees and produces cheaper cocoa beans is the *Forastero* tree used for about 80% chocolate in the globe. The Arriba, considered to be the best of the other types has significance health benefits (Rusconi & Conti, 2010; Ovellanos *et al*., 2007). Cocoa is linked to human health and different systems including cardiovascular, neurological, oral health, endocrine, lymphatic and immunological, respiratory, reproductive, respiratory and dermatological that constitute the human body (Araujo *et al*., 2016). Cocoa has been used for a number of years as a medicine to fight against inflammation, pain, treat upper respiratory tract conditions involving colds and coughs (Selmi et al., 2008; Tomaru *et al*., 2007), enhance mental well-being, protection against nutritional deficiencies and several other ailments due to its rich source of flavonoid and theobromine (Ludden & Moore, 1998).

Cocoa contains over 300 different constituents comprising cocoa butter (oleic, palmitic and stearic fatty acids), minerals (magnesium, iron, potassium and zinc), methylxanthine (theobromine and caffeine), polyphenols including other compounds involving tyramine, tryptophan, and serotonin (Wollgast & Anklam, 2000). Cocoa beans and their byproducts such as cocoa powder and chocolate are important sources of polyphenols (Katz *et al*, 2011; Lamuela-Raventós *et al*., 2005). Cocoa is a critical source of dietary polyphenols which contains specific antioxidants flavonols including epicatechin, catechin and quercetin which are structurally comparable to the antioxidants in grapes and tea (Noori *et al*, 2009).

The extensive polyphenol class comprise flavonoids obtained from cocoa beans. The key components of flavonoid are flavan-3-ols, occurring as monomeric (-)-epcatechin and (+) -

catechin, together with type-B proanthcyanidins, formed from monomeric flavanols (Ellam & Williamson, 2013). Cocoa procyanidins include B2 and B5 dimers and the C1 trimmer together with high levels of longer-chain polymers encompassing four or more monomeric units (Gu *et al*., 2006).

Cocoa contain theobromine, a 3, 7-dimethylated xanthine alkaloid also formed during metabolism of caffeine. About 2.5% of dry weight of cocoa contain high levels of theobromine. Theobromine can be used as a marker of cocoa content (Risner, 2008). It is mostly consumed as dietary supplement from cocoa and its high bioavailability as well as potential biological activities suggest its use as interventional agent in most studies (Dock, 1926). Magnesium is found at significant levels in cocoa about 2-4 mg/g dry powder according to the US Department of Agriculture's National Nutrient Database. Magnesium is a critical cofactor in several enzyme-catalysed reactions in vivo. It is essential for maintenance of blood pressure, muscular contraction and neuronal transmission (Ellam & Williamson, 2013).

2.11.2 Bioavailability of Cocoa flavonoids

Consumption of cocoa products are metabolize in the form that any biologically active constituents reach target tissues. Salivary proteins in the mouth bind to procyanidins influenced by interflavan-3-ol and chemical nature of monomeric units rather than size of the oligomers (Freitas & Mateus, 2001; Bacon & Rhodes, 2000). Flavanols are stable in the stomach and able to reach the small intestines where epicatechin is absorbed (Giuffrida *et al.*, 2012). According to Kida *et al*., (2001), flavan-3-ol monomer conjugates are transferred to the liver from the bloodstream and return to the small intestine through the bile. Both human and animal studies showed that procyanidin dimer B2 is poorly absorbed as detected in the urine after consumption (Urpi-sarda *et al*., 2009). A study conducted by Ellam & Williamson, (2013) indicate that flavanol oligomers with high molecular

weight reach the small intestine intact and available for absorption. Absorption in the small intestine of dimers and trimers are less efficient compared to epicatechin and catechin monomers. Enzymes such as lactase, phloridzin hydrolase and cytosolic β-glucoridase in the small intestine hydrolysed some glycosides (Ellam & Williamson, 2013)

Dietary polyphenols represent the main source of antioxidants for humans use (Food *et al*., 2005). Metabolism and absorption of phenolic compounds depend on certain factors including chemical structure, molecular size and solubility which are associated with the degree of glycosylation, acylation or polymerization (Goya *et al.*, 2016). Cocoa polyphenols have relatively low bioavailability and their activity indicates that epicatechin is much better absorbed than catechin due to stereochemical differences resulting in different degrees of hydrophobicity (Steinberg *et al*., 2002). Epicatechin metabolites, glucuronide and 3-O-methylglucuronide can cross the blood-brain barrier and act at the cerebral level. The metabolites of theobromine such as methyl xanthine and methyl uric are extensively absorbed in the small intestine, diffuse passively via the enterocytes into the hepatic circulation (Steinberg *et al*., 2002).

2.11.3 Health benefits of natural cocoa in HFD fed animals

Cocoa has numerous benefits spanning from anti-aging to anti-inflammatory properties (Ellinger & Stehle, 2016; Kim *et al.*, 2014). Cocoa is a good source of magnesium, copper, sulphur, calcium, zinc, potassium, iron, manganese, and vitamin B (Scapagnini *et al.*, 2014; Katz *et al*., 2011). It is therefore suggested that, the antioxidant and anti-inflammatory properties in cocoa will play an important role in attenuating obesity and its related disorders in high-fat fed rats. The polyphenols in cocoa have anti-obesity effect due to its capacity to suppress fatty acid synthesis and stimulating cell energy expenditure in the mitochondria (Andújar *et al*, 2012; Matsui *et al*., 2005). Oxidative stress is considered a major cause of molecular injury to several cells which results from difference

in free radicals and antioxidants in the body and have been involved in the pathogenesis of numerous diseases comprising the progression of hepatic diseases and damages to fat cells (Martín *et al.*, 2010). Many compounds as well as plants containing antioxidant properties have the capacity to nullify the disorders of oxidative stress (Martín & Ramos, 2016). Natural polyphenolic plants especially flavonoids from cocoa with their strong antioxidant properties have been confirmed to have therapeutic potentials for certain human conditions such as obesity, insulin resistance atherosclerosis, NAFD, type 2 diabetes, (Martín & Ramos, 2016; Martín *et al.*, 2010).

Polyphenols and methyl xanthine in cocoa contribute to improved insulin sensitivity, decreased both fasting and postprandial glucose levels and the flavonoids in cocoa has been identified to boost metabolism aiding the body to metabolize fat better to reduce weight (Rowley, 2017; Martín *et al*., 2014; Medicine *et al*., 2012; Martín *et al*., 2010; Jalil *et al*., 2007; Brand-miller *et al*, 2007) and enhance postprandial insulin secretion (Rowley *et al*., 2017). The theobromine in cocoa according to Coronado-Cáceres *et al*. (2019) and Jang *et al*. (2015) has capacity to decrease the mass of mesenteric and epididymal fat in HFD animals. Data demonstrate that oligometric procyanidins in cocoa have the capacity in preventing weight gain from high-fat intake, maintaining fasting glucose, insulin levels, glucose tolerance, improving insulin sensitivity, normalize blood glucose level and reduce adipose tissue mass (Dorenkott *et al*., 2014). Cocoa epicatechin monomers help the pancreatic β cells to secrete better insulin and respond more efficiently to increased blood glucose (Rowley *et al*., 2017). Several studies have proven that the consumption of flavonoids including cocoa and cocoa products flavonoids reduce the risk of heart diseases (Borchers *et al*., 2000). Further studies showed regular consumption of natural cocoa enhanced wound healing in rabbits, ameliorated liver injury in plasmodium infection (Addai *et al.*, 2012) and reduced alcohol toxicity in rats (Sokpor *et al.*, 2012). The flavonoids in cocoa has

beneficial capacity on inflammatory activity as well as being cancer-protective agents (Borchers *et al*., 2000). Cocoa flavonoids have the capacity to reduce platelet activation *in vivo* which can lead to atherosclerosis (Rein *et al*., 2000). The polyphenolic-rich cocoa extract contain hypoglycemic and hypocholesterolemic effects reducing glucose and cholesterol level in the blood (Ruzaidi et al., 2008) and suppresses hepato carcinogenesis (Meng *et al*., 2009). The intake of flavonoid-rich cocoa have a very low incidence of ischemic heart diseases, stroke and hypertension (Bayard *et al*, 2007).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Ethical Approval

Ethical clearance was sought from the University of Ghana Medical School, College of Health Sciences (CHS), Korle-Bu, Ethical and Protocol Review Committee (EPRC). A protocol Identification Number CHS-Et/M3-5.1/2019-2020 was issued for this study.

3.2 Pilot Study

A pilot study was conducted at the Animal Experimental Unit of the University of Ghana Medical School for nine (9) weeks to optimize treatment protocol for the main study. Two different sources of fat (coconut oil and lard) with different percentages (20% and 50%) were used. Six (6) Sprague-Dawley rats aged 14-15 weeks and weighing between 235-310 g were grouped into three of two animals each. Group 1 (control) fed on the standard chow and tap water. Group 2 fed on high-fat diet (20% of coconut oil) and served with tap water. Group 3 fed on high-fat diet (20% of lard) and served with tap water. Another six (6) Sprague-Dawley rats aged 14-15 weeks and weighing between 235-310 g were grouped into three of two animals each. Group 1 (control) fed on the standard chow and tap water. Group 2 fed on high-fat diet (50% of coconut oil) and served with tap water. Group 3 fed on high-fat diet (50% of lard) and served with tap water.

During the experimental period, body weight of all rats in the various groups was monitored weekly as well as feed intake and fluid intake were measured daily. At the end of 9 weeks of treatment, rats were sacrificed by and their livers and adipose tissues (peri-intestinal, mesenteric and epididymal) were harvested for histological examination. Qualitative histological observation

of micrographs revealed morphological changes of adipocytes and hepatocytes in both of the high-fat diet (20% and 50% of both coconut oil and lard).

Rats that consumed 50% fat both in coconut oil and lard groups had rough and moist fur with reduced weight. Rats that consumed 20% fat both in coconut oil and lard groups had smooth fur with minimal weight gain. With these observation the fat content of 40% was used in the main study. Also, coconut oil was used in the main study due to its availability and ability to induce adipocyte hypertrophy and fat droplet infiltration of the liver.

3.3 Experimental Protocol for main study

3.3.1 Study design

The design for the study was experimental

3.3.2 Study site

The study was conducted at the Animal Experimental Unit of the University of Ghana Medical School, College of Health Science, Korle-Bu. The unit has a laboratory with ambient temperature of 28 ± 2^{o} C, relative humidity of (70 ± 2) %. The unit has about 80 small cages (28.7cm length x 20.3cm width x 17.3cm height) each of which can accommodate two rats and big cages (52.8cm length x 48.3cm width x 26.2cm height) each of which can accommodate eight to ten rats. The laboratory also has tap water supply, and it is run on an alternating 12-hour period of light and 12-hour period of darkness.

3.3.3 Study population

Twenty four (24) male Sprague-Dawley rats aged 10-12 weeks and weighing between 195-230g were used for the study.

3.3.4 Acquisition and acclimatization of animals

The rats were purchased from Animal Laboratory Unit of the Centre for Plant Medicine Research (CPMR), Mampong-Akuapem in the Eastern Region of Ghana. All animals were kept and acclimatized for one week by monitoring their weight and feed intake. There were consistency in their weight and feed intake after one week acclimatization period. The rats were then weighed and randomly divided into four (4) groups. Procedures involving the animal and their care conformed to the institutional guidelines, in compliance with National and International regulations and guidelines for the use of animals in biomedical research.

3.3.5 Animal grouping and administration of treatments

After one week acclimatization period, the rats were assigned into four (4) main groups of six (6), making sure that their mean body weights were not significantly different using Bartlett test of variance. Cages (dimension: 52.8cm length x 48.3cm width x 26.2cm height) were used to house each group of rats. Their weights were recorded on the first day of treatment as the baseline for each group.

The twenty four (24) male Sprague-Dawley rats were grouped into four. Group one (G1) the control group, received standard rat chow and had free access to clean tap water. Group two (G2) animals received standard rat chow and free access to 2% w/v of natural cocoa drink. Group three (G3) rats received high-fat diet (HFD) and free access to clean tap water and group four (G4) received HFD and free access to 2% w/v of natural cocoa drink. The summary of groupings and treatments are shown in figure 5. The amount of feed and the volume of fluid consumed every day (24hours) were recorded for each group. The experimental treatment lasted for nine (9) weeks during which the rats were weighed weekly and their weight recorded.

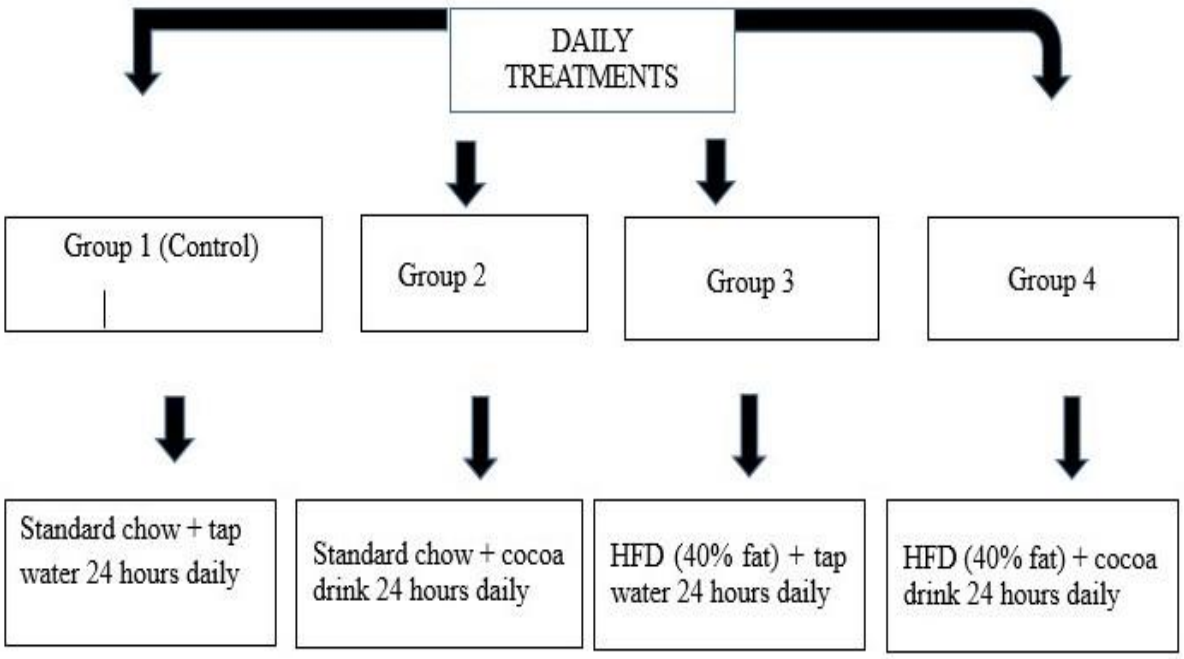


Figure 5. Diagram summarizing the groupings and daily treatments of rats.

3.3.6 Preparation of high-fat diet (HFD)

620g of the rat chow was measured using electric balance and 380ml of coconut oil was measured using measuring cylinder. The rat chow (5% of fat) and the coconut oil was mixed together. The mixture was fried for about 10 minutes to prevent the feed from going rancid (Lima & Block, 2019). The summary of the composition of the HFD is shown in table 1.

Coconut oil is a colourless to brown-yellow edible oil derived from mature and dry coconut meat. The oil is composed of fatty acids (99.9%) of which the content of saturated fatty acids is 91.9%, monounsaturated fatty acids constitute 6.4% and polyunsaturated fatty acids being 1.5% (England, Public Health, 2019). The major fatty acids found in coconut oil include lauric, myristic and palmitic acids (Boateng *et al*, 2016).

Table 1: Summary of high-fat diet (HFD) composition

Formulation	nutrients	Analysis (%)
620g of feed + 380 ml of coconut oil	Dry mass	0.000
	Crude protein	10.634
	Ether extract (fat)	40.000
	Crude fibre	2.077
	Lysine	0.248
	Methionine	0.472
	Tryptophan	0.000
	Salt	0.347
	Energy	5133.381
	Carbohydrate and protein	45.533

3.3.7 Preparation of 2% (w/v) unsweetened natural cocoa drink

Unsweetened natural cocoa drink was prepared at a concentration of 2% w/v of natural cocoa powder (GoodFood® brand, Ghana) as described in the works of Sokpor et al. (2012) and Addai et al. (2012). The cocoa drink was prepared daily by weighing 5g of cocoa powder with a chemical weighing balance (Mettler Teledo P1200, Switzerland) and thoroughly dissolved by stirring in 250

ml of freshly boiled tap water contained in a beaker. The cocoa suspension was poured into a graduated feeding bottle after it was allowed to cool and then positioned at a reachable point of the rat cage as shown in Figure 6. To prevent sedimentation of particles of the cocoa suspension, the cocoa suspension feeding bottle was agitated at every 4 hours. This was done to prevent blockage of the inverted feeding bottle teat and allowed free flowing of the cocoa drink when sucked by the rats. Groups 2 (G2) and 4 (G4) were served with the cocoa drink and groups 1 (G1) and 3 (G3) received tap water throughout the experimental period as shown in figure 6.

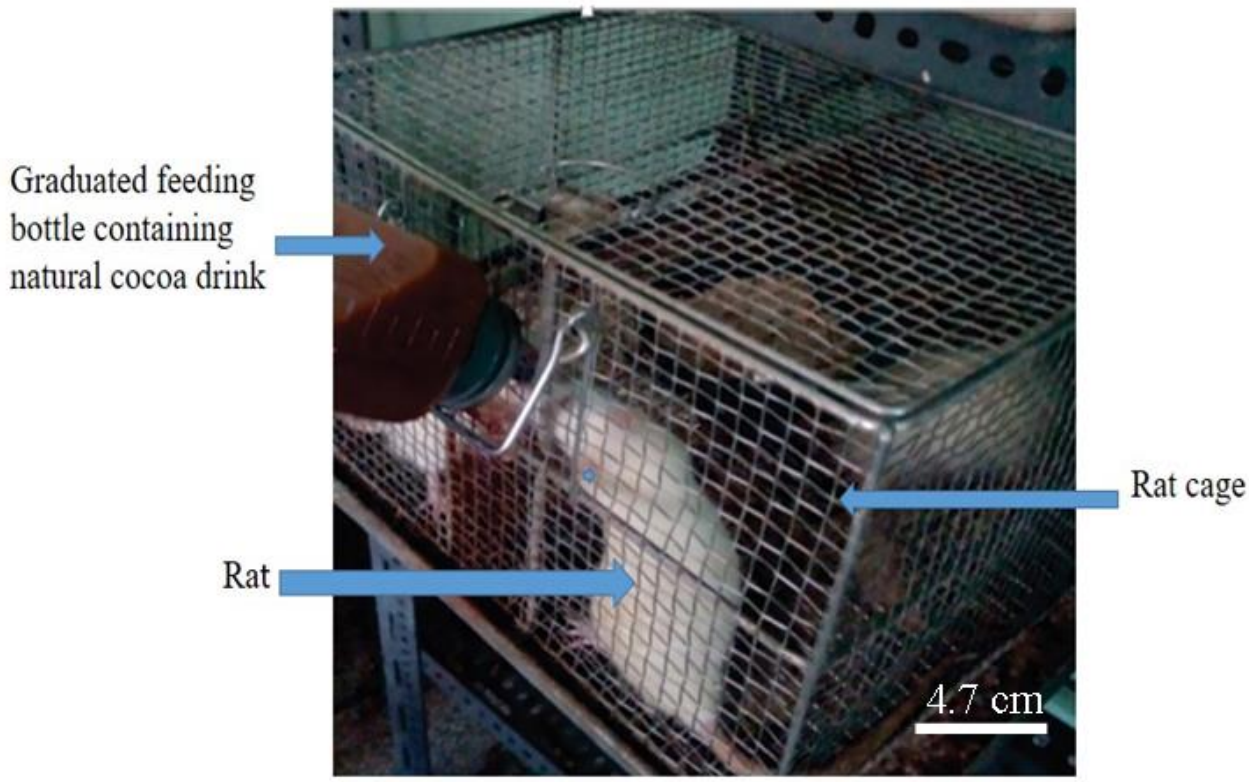


Figure 6. Photograph showing a rat voluntarily consuming freshly prepared NCD suspension in a graduated feeding bottle.

3.4 Assessment of variables

3.4.1 Determination of weight of rats

The weight of the rats in the various groups were measured weekly and was done in the morning. The weight of the experimental rats was determined using a weighing scale (TS-872, TGC, Japan).

3.4.2 Determination of feed and fluid (cocoa drink and tap water) consumed by rats

The volume of natural cocoa drink and tap water as well as the weight of feed (normal rat chow and high-fat diet) consumed by each group was measured daily. The measurement was done every 24 hours by deducting the amount of feed left in the feeding trough from the initial amount of feed administered. Groups one (G1) and two (G2) were exposed to standard rat chow (SRC). To calculate the amount of feed consumed in these groups, the final standard rat chow (SRCf) was deducted from the initial standard rat chow (NRCi). Groups three (G3) and four (G4) were exposed to high-fat diet (HFD). To calculate the amount of feed consumed in these groups, the final high-fat diet (HFDf) was deducted from the initial high-fat diet (HFDi). Groups two (G2) and four (G4) received natural cocoa drink (NCD). Groups one (G1) and three (G3) took tap water. To calculate the volume of cocoa drink consumed, the final natural cocoa drink (NCDf) was deducted from the initial natural cocoa drink (NCDi). The volume of water consumed was determined by deducting the final volume of water ($Vol._F$) from the initial volume of water ($Vol._I$).

The volume of fluid left was subtracted from the initial volume to obtain the amount of fluid consumed. Also, the weight of feed left was deducted from the initial weight to obtain the amount of feed consumed.

3.4.3 Determination of fasting blood glucose (FBG) levels

The glucose levels of the experimental rats were determined weekly and was done in the morning. Rats were fasted for eight (8) hours. The FBG was determined using a drop of blood obtained from the rat's tail tip. About 1-3mm incision was made using dissecting blade fixed on a scalpel. This was done in accordance with the Office of Ethics and Compliance Institutional Animal Care and Use Program. The glucometer strip (Safe-Accu 2 kit, Germany) already inserted into the glucometer machine was used to measure the glucose level from the drop of blood from the rats. The readings of the FBG were recorded.

3.4.4 Determination of oral glucose tolerance test (OGTT)

Oral glucose tolerance test (OGTT) was performed to assess disturbances in metabolism of glucose that can be associated with obesity, metabolic syndrome and diabetes. The OGTT measures the clearance of an oral glucose load from the body (Metabolic & Centers, 2016). Two days before sacrificing the animals and terminating experiment, an oral glucose tolerance test using glucose 2g/Kg body weight was performed after 8 hours fasting period. A solution of glucose (20% dextrose) was administered by oral gavage as described by (Metabolic & Centers, 2016). Blood glucose concentration was measured using glucometer (Safe-Accu 2 kit, Germany) at 0, 30, 60, 90 and 120 min.

3.4.5 Collection of blood samples for biochemical analysis

Blood samples were taken through cardiac puncture after the animals had been anaesthetized by diethyl ether inhalation at weeks 0 and 9. Blood samples for week 0 served as baseline data before treatment commenced. The samples collected (1.5 ml) were allowed to stand for 30 minutes to speed up clotting process and serum separation. The samples were appropriately labelled and spun at 3500 revolutions per minute (rpm) for seven (7) minutes using a high-speed refrigerated

centrifuge (TGL-16MC) in the Medical Biochemistry Department, UGMS, Korle-Bu. The serum obtained was then transferred into 5ml Eppendorf tubes and stored at -20^{O}C. Samples were later used for biochemical assay of inflammation marker TNF-α and in the serum. The same procedures were repeated for rats at the end of the 9th week of the experimental treatment.

3.4.6 Tumor Necrosis Factor alpha (TNF-α) concentration the blood serum

Concentration of TNF- α was measured using the TNF-α ELISA Kit (Biomatik Corporation, USA with item number; BK EKA51931 from South Africa) according to the manufacturer's protocol. The assay was performed at Noguchi Memorial Institute for Medical Research in accordance with the guidelines set out by the reagent manual. Endpoint absorbance was read by spectrophotometer at a 405 nm after thirty (30) minutes of incubation at room temperature. The TNF-α activity was then calculated by the formula provided by the protocol manual.

3.5 Harvesting of tissues and histological processing

3.5.1 Harvesting of tissues

Rats were transported from the animal experimentation unit to the histology laboratory at the Department of Anatomy, University of Ghana Medical School at the termination of the experiment at the end of the 9th week to be euthanized. A standardized perfusion set-up at the laboratory was used for the perfusion. A clean improvised desiccator without any desiccant was used as a chamber where rats were anaesthetized and sacrificed. A cotton wool soaked with diethyl ether was placed in the desiccator and covered tightly. Rats were placed in the desiccator until they lost consciousness which was evidenced by pin-pricking the foot without consequent movement and pain. The rat was quickly removed and pinned on a clean dissecting board in a supine position as shown in figure 7. The thoracoabdominal region was cleaned with 70% alcohol prior to dissection.

Incisions were made at the anterior wall of the thoracic region and extended to the abdominal cavity. Incisions were reflected superiorly to expose the thoracic and abdominal viscera.

Normal saline (0.9%) was used to perfuse and flush the cardiovascular system of the rat. Perfusion was done using 21G ×1" hypodermic needle inserted into the left ventricle of the rat. The right atrium was nicked with a pair of scissors to serve as an outlet for the 0.9% normal saline and fixatives. A satisfactory clearing was established by the bright red appearance of the liver becoming pale as well as the flow of the 0.9% normal saline through the nostrils.

The animals were then perfused with about 250 ml of 10% neutral buffered formalin (pH=7.24-7.28) using the same standardized set up. Effective fixation was confirmed by stiffening of the animal after all muscles had stopped twitching. Adipose tissues from the epididymis, intestines and mesentery of each animal were carefully dissected out as shown in figure 8.

The harvested adipose tissues were divided into two. One half was selected, fixed in 10% neutral buffered formalin and stored in -4^{o}C overnight before tissue processing for light microscopy commenced.

The liver was also carefully dissected out after perfusion as shown in figure 8, weighed on an electrical balance (Mettler P1200-Switzerland) and the weight recorded. Each of the four lobes (right, left, middle and caudate or papillary) harvested were divided into three. The liver tissues were post-fixed in 50 ml of 10% buffered formalin (pH 7.4) for a week before tissue processing for light microscopy began.

3.5.2 Rat liver volume determination

Liquid displacement method was used to determine the volume of the liver. For each measurement, a measuring cylinder was filled with 50 ml of water and the initial volume recorded. The liver was

gently placed and totally immersed in the measuring cylinder and the final volume was recorded. The difference between the final volume and the initial volume was recorded as the volume of the whole liver. The liver was then quickly transferred into another container containing the fixative (10% buffered formalin) for fixation and subsequent tissue processing.

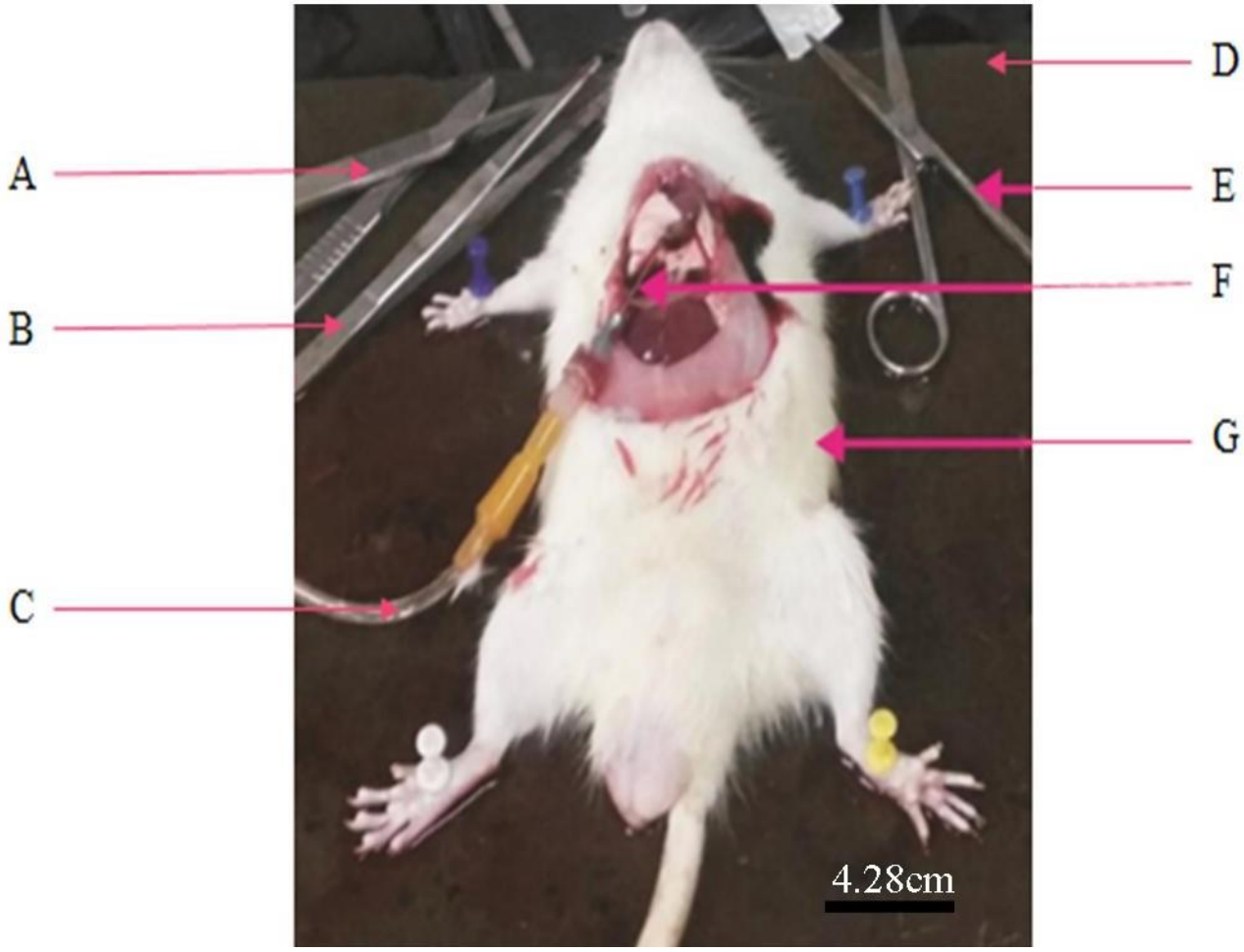


Figure 7. Photograph of a rat undergoing perfusion. A=Scalpel with blade, B=Forceps, C=Infusion line, D=Dissecting Board, E=Pair of Scissors, F= 21Gx1 Hypodermic Needle, G=Rat

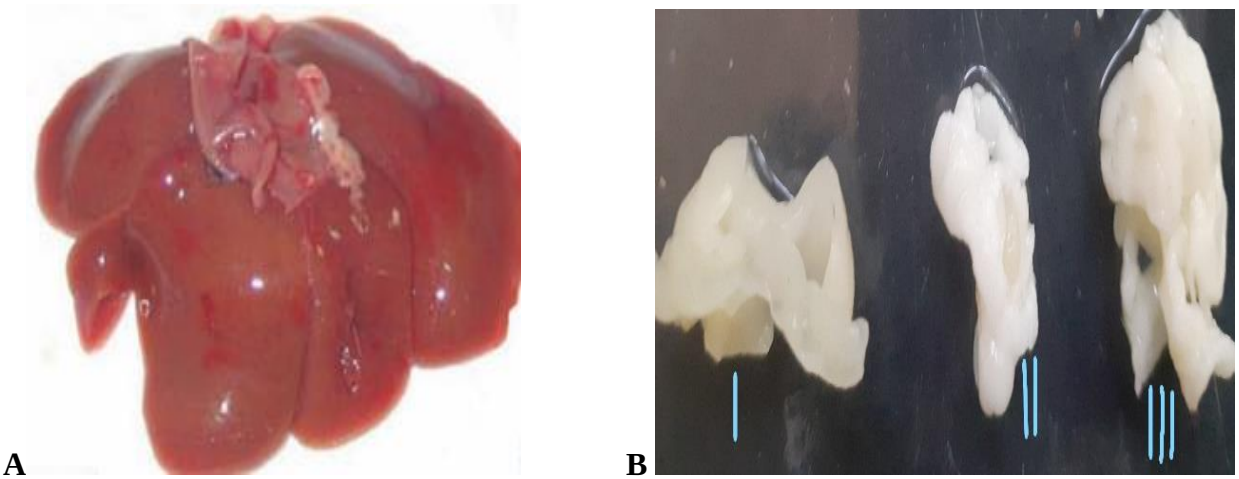


Figure 8. Pictures showing perfused liver and adipose tissue harvested from the experimental rats after the experimental period (9 weeks). A = Liver, B = Adipose tissues (I = Epididymal, II = Intestinal and III = Mesenteric).

3.5.3 Slicing and processing of liver tissue for histological assessment

Each of the four lobes (right, left, middle and caudate or papillary) of the liver was cut longitudinally into three slices. To systematically select a slice for processing, the second slice of each lobe was selected for histological processing. As a result, four slices were obtained from each rat in a group and separately processed. The sliced liver tissues were processed by following the routine histological protocols. Sliced liver tissues were placed in tissue cassettes and were dehydrated through graded series of alcohol from 50%, 70%, and 95% to absolute), cleared with series of xylene and were further infiltrated with molten wax (embedded) to form blocks for sectioning into desired thickness (5µm) using a microtome.

3.5.4 Slicing and processing of adipose tissues

The harvested adipose tissues (epididymal, intestinal and mesenteric) were stored in -4^{o}C overnight. Each of the tissues was cut into two slices and one randomly placed in a tissue cassette.

The cassettes containing the tissues were washed in 50% ethanol and kept in another 50% ethanol overnight. They were then passed through series of graded ethanol followed by xylene before infiltrating with molten wax for embedding. The tissues blocks were sections into desired thickness (5µm) using a Leica microtome (Leica RM 2125, Germany).

3.5.5 Sectioning of tissues (liver and adipose)

Each tissue block was carefully trimmed at 10µm thickness using the Leica microtome (Leica RM 2125, Germany). After the trimming, the liver and the adipose tissues were sectioned at 5µm. Two sections of liver tissues were systematically selected from blocks consisting of all the lobes per rat for Haematoxylin and Eosin staining. The two sections were selected at every 10^{th} and 100^{th} sections. For each rat, a total of eight (8) liver sections were obtained and mounted on glass slides (76 x 26mm x 1mm).

Two sections were systematically selected from each of the epididymal, intestinal as well as mesenteric adipose tissues block per rat. The two sections were selected at every 5^{th} and 50^{th} sections. For each rat, a total of six (6) adipose tissue sections were obtained and mounted on glass slides (76 x 26mm x 1mm). In all a total of six slides were obtained per rat. Photomicrographs were taken from the slides for stereological assessment.

3.6 Histological and morphometric assessment

3.6.1 Sampling of photomicrographs of liver and adipose tissues sections.

The volume density of fat infiltrations in hepatocytes and diameter of adipose tissue were determined to assess the morphological alterations among the experimental groups. A bright field binocular light microscope (Leica Gallen III, catalogue no. 317506, serial no. ZG6JA4) was used to examine the liver and the adipocyte slides. To capture micrographs of liver tissues, a random starting point was chosen and after that the microscope stage was moved three graduations on the

X-axis and two graduations on the Y-axis of the Leica Gallen III microscope stage unit intervals. To capture micrographs of adipose tissue, the stage was moved two graduations in the X-axis and one graduation in the Y-axis of the Leica Gallen III microscope stage unit intervals. The stage of the microscope was moved from one plane on the X-axis to a different plane on the Y-axis. Using the x40 objective lens and a digital eyepiece (Lenovo Q350 USB PC Camera) attached to the computer (HP Compaq dx2300 Microtower), photographs of fields of view under the microscope as determined by the X and Y axes movements were captured. This procedure was done until the whole area of each section was covered.

3.6.2. Stereological analysis of the liver

The volume density of fat droplets that had infiltrated hepatocytes was determined by employing Cavalieri's principle with point counting. A grid of dimension 1cm x 1cm in Adobe Photoshop CS6 software Extended (trial version 13.0.1) was superimposed on each micrograph of the liver tissue as shown in figure 9. Parameters such as fat infiltrations (fat droplets) as shown in figure 9 in the hepatocytes were counted at the point of intersection of the grid lines. Values from the point counting were entered into the formula (Cavalieri estimator of volume) below for the calculation of relative volume density.

$$Vv = \frac{\Sigma P \times (\frac{a}{p}) \times t}{M^2}$$

Where Vv indicates volume density, ΣP is the sum of all test points encountered, shown in figure 9 as red solid arrows, (a/p) is the area per point of the stereological grid, t is the thickness of the section and M is the linear magnification.

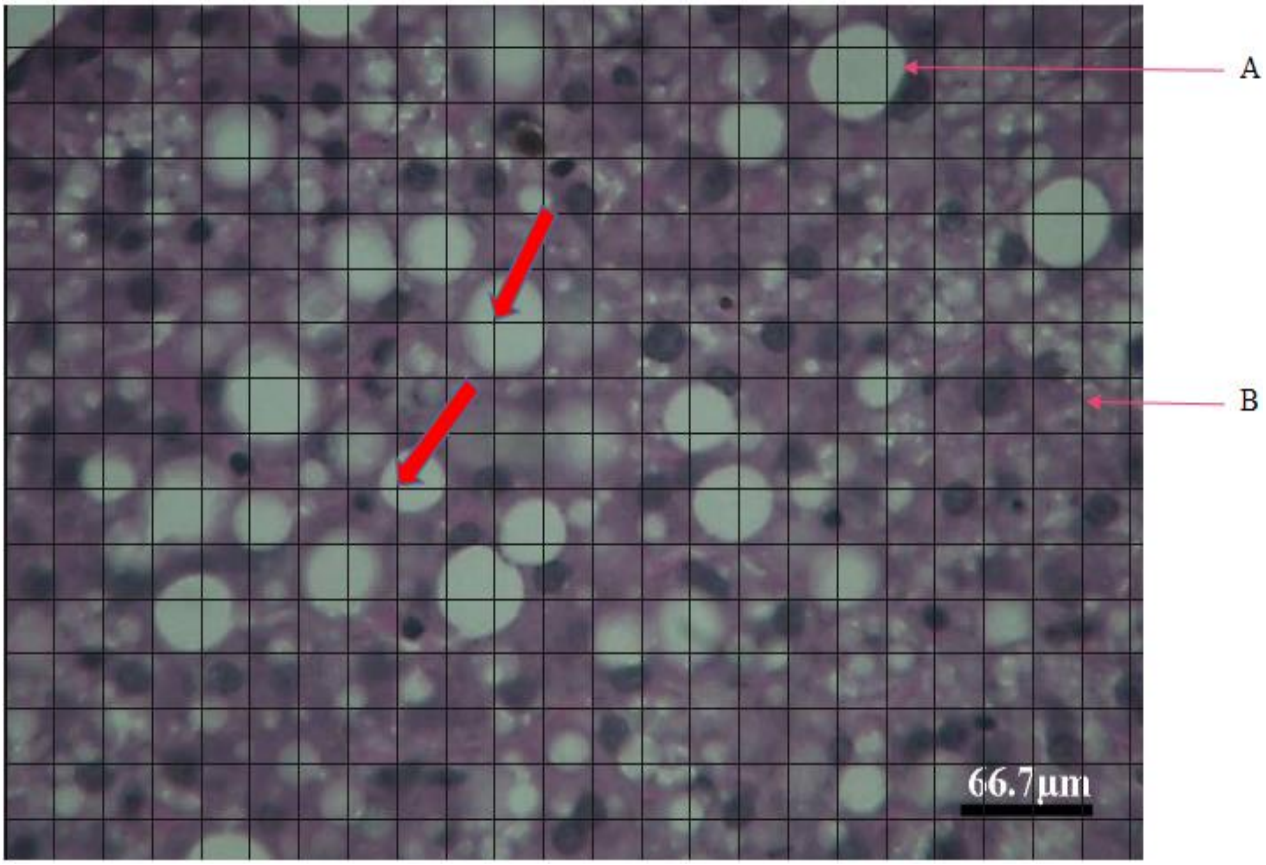


Figure 9. Picture showing test grid with square lattices superimposed on liver photomicrograph for stereological estimation of fat infiltration. A is fat droplet, B is test grid and Red solid arrows pointing to test point hitting fat droplet.

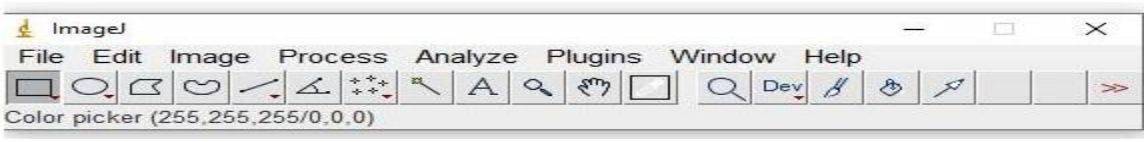
3.6.3. Determination of adipocyte size in adipose tissue

The adipocyte sizes were determined using an ImageJ software as shown in figure 10A. The software was calibrated using a micrograph of the stage graticule captured with the x40 objective lens. The stage graticule was uploaded onto the ImageJ software. A ruler tool was used to draw a straight line from 0µm to the 100µm on the graticule. The value obtained was the distance in pixels and the known distance was 100µm (i.e. distance between 0-100µm on the graticule). The unit was

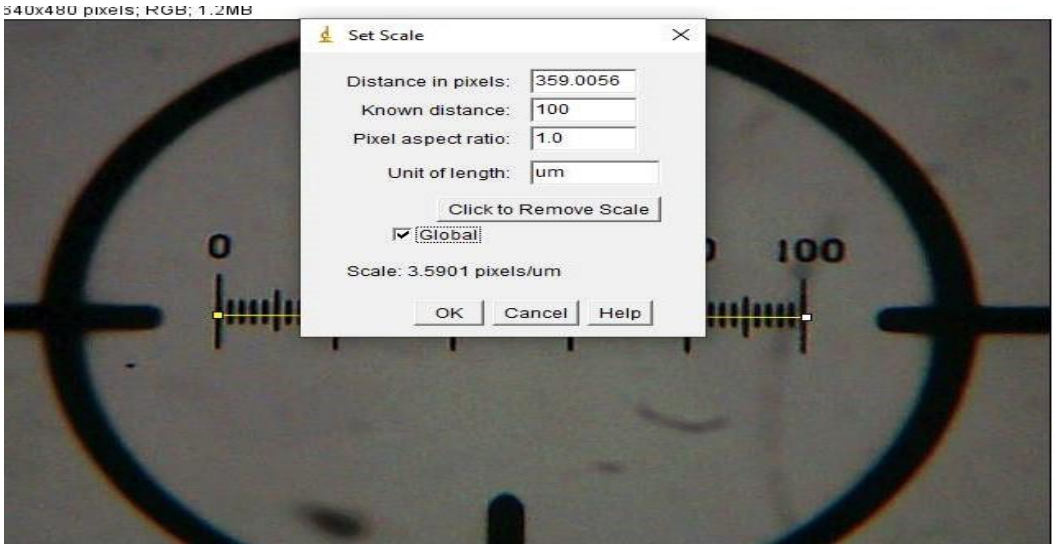
set to micrometers (μm) to give a scale factor and global on the scale was checked to apply to all the adipocytes on the photomicrographs to be measured as shown in figure 10B. Afterward, the adipocytes micrographs were individually uploaded onto the software. Using the ruler tool, a line was drawn along the widest diameter of a selected adipocyte. Automatically the software generated and recorded the diameter as shown in figure 10D. Two adipocytes were randomly selected from the photomicrographs and their sizes (diameters) measured as shown in figure 10C. This procedure was done until all photomicrographs of adipose tissues (epididymal, intestinal and mesenteric) in the various groups were measured. The average of the sizes of the adipocytes in the various groups were computed.

3.7 Statistical analysis

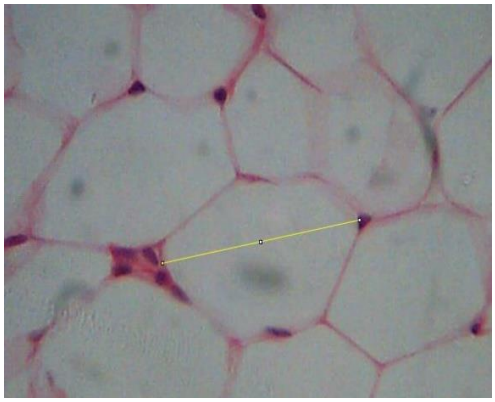
The Graph Pad Prism software (version 5) was used for the analysis. All results were expressed as means and standard deviation, as well as 95% confidence interval (Cl) for means were used. One-way ANOVA and Tukey's post hoc test were used to compare the means within and between the treatment groups. The *p-value* ≤ 0.05 was considered statistically significant. Test for homogeneity of variance was done using Bartlett's test for equality of variances.



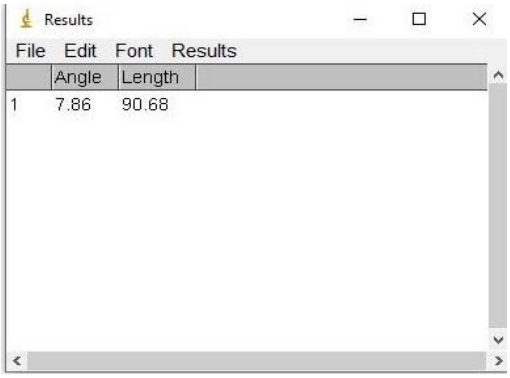
A



B



C



D

Figure 10. Pictures showing how adipocyte sizes were measured. A is showing the ImageJ software interface, B is showing how the software was calibrated using a micrograph of the stage graticule, C is showing a micrograph of a slide of adipose tissue uploaded onto ImageJ and whose widest diameter was being determined using the free hand line drawing tool and D is displaying the measured size (diameter).

CHAPTER FOUR

4.0 RESULTS

4.1 Effect of HFD and natural cocoa on some assessed variables

4.1.1 Effect of HFD and natural cocoa on the weight of animals

There were four groups in this study. The groups and the treatments for the various groups are described below:

Group one (G1) also the control, fed on standard rat chow and tap water. Group two (G2) fed on standard rat chow and treated with cocoa. Group three (G3) was treated with HFD only. Group four (G4) fed on HFD treated with cocoa.

Figure 11 represents the weekly mean weight of rats and between group comparisons using one-way ANOVA followed by Tukey's post hoc test. The line plot graph shows that, the rats in the various groups gained weight throughout the experimental period (9 weeks) but to a larger extent in the HFD only (G3) group. There was significantly decreased weight gain observed in the HFD treated with cocoa (G4) group while the control (G1) and the HFD only (G3) groups had increased weight gain in week five (5). Also, there were statistically significant differences in weight gain observed in weeks six (6), seven (7), eight (8) and nine (9) between HFD treated with cocoa (G4) when compared to HFD only (G3) group. This implied that there were decreased in weight gain observed in HFD treated with cocoa (G4) group whereas the HFD only (G3) group had increased weight gain. Similarly, there were statistically significant differences in weight gain observed in weeks eight (8) and nine (9) between HFD treated with cocoa (G4) when compared to the control (G1). This implied there were significantly decreased weight gain in HFD treated with cocoa (G4)

group while the control (G1) group had increased weight gain with p-values 0.0183 and 0.0167 in weeks eight (8) and nine (9) respectively.

From figure 11, it indicates that HFD induced increased weight gain in rats in the HFD only (G3) group when compared to other groups while rats given natural cocoa in G4 and G2 showed decreased weight gain. This suggests that HFD increased weight gain and natural cocoa intake decreased weight gain in HFD fed rats.

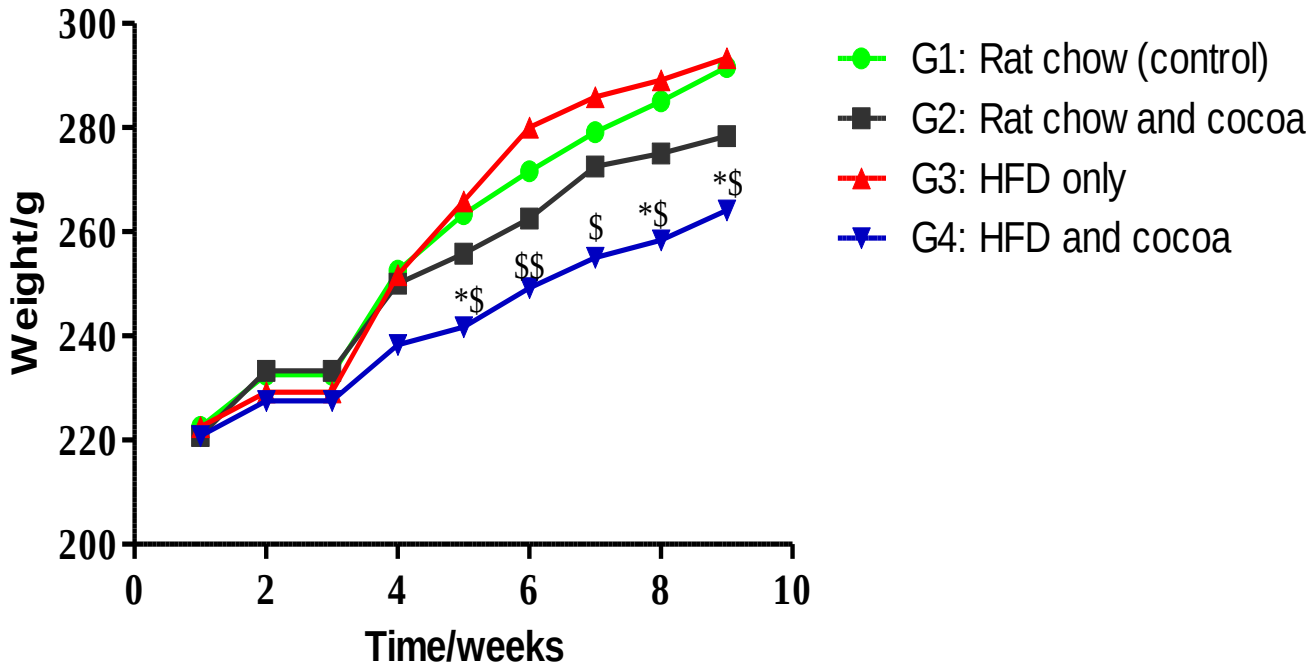


Figure 11. Line plot showing weekly mean weight of rats during the experimental period. Each point represents mean weight of rats in the group for specific week and *p-value* represents significance level for one-way ANOVA (followed by Tukey's post hoc) for between-group comparison with * and $ indicating level of statistically significant difference compared to G1 and G3 respectively. *P<0.05 and $P<0.05, $$P<0.01.

4.1.2 Feed consumption of the experimental groups

Table 2 shows the weekly mean feed consumption and between group comparisons using one-way ANOVA followed by Tukey's post hoc test. There were statistically significant differences in amount of feed intake from week one (1) to week four (4) between both HFD only (G3) and HFD treated with cocoa (G4) groups when compared to both the control (G1) and the standard chow treated with cocoa (G2) groups. This indicates significantly less amount of feed intake in both the HFD only (G3) and HFD treated with cocoa (G4) groups and more amount of feed intake in both the control (G1) and standard chow treated with cocoa (G2) groups. Also, there were significantly less amount of feed intake in the HFD treated with cocoa group (G4) and more amount of feed intake in both the control (G1) and the standard chow treated with cocoa (G4) groups in week five (5). Week six (6) recorded significantly less amount of feed intake in both HFD only (G3) and HFD treated with cocoa (G4) groups and more amount of feed intake in both the control (G1) and the standard chow treated with cocoa (G2) groups. In weeks seven (7) and eight (8), there were significantly less amount of feed intake in the HFD only (G4) group and more amount of feed intake in both the control (G1) and the standard chow treated with cocoa (G2) groups.

This suggests that the control (G1) and the standard chow treated with cocoa (G2) groups consumed more amount of feed than the HFD only (G3) and the HFD treated with cocoa (G4) groups. The amount of feed intake in the control (G1) and the standard chow treated with cocoa (G2) was similar and that of HFD only (G3) and HFD treated with cocoa (G4) was also similar.

Table 2. Weekly mean feed intake of rats in grams during the experimental period. Values are means with standard deviation in brackets

Animal groups	G 1	G 2	G 3	G 4	P-value 1
Week 1	145.00 (12.5)	160.00 (18.2)	124.28 (9.7) $^{*\#\#\#}$	112.14 (10.7) $^{***\#\#\#}$	0.0001
Week 2	175.00 (7.6) ^{a}	170.00 (13.8)	135.71 (13.9) $^{***\#\#\#}$	144.28 (12.7) $^{***\#\#c}$	0.0001
Week 3	160.00 (11.5)	170.00 (14.1)	132.85 (11.1) $^{**\#\#\#}$	128.57 (13.4) $^{***\#\#\#}$	0.0001
Week 4	160.00 (15.2)	165.71 (18.1)	130. 71 (16.4) $^{**\#\#}$	128.57 (10.6) $^{**\#\#\#}$	0.0001
Week 5	167.14 (21.3)	174.28 (24.3)	155.00 (20.6) ^{b}	134.28 (12.3) $^{*\#\#a}$	0.0062
Week 6	151.42 (15.7)	154.28 (18.1)	130.00 (10.0) $^{*\#}$	118.57 (8.9) $^{***\#\#\#}$	0.0001
Week 7	165.71 (16.1)	168.57 (14.6)	155.71 (12.7) ^{b}	147.14 (13.8) $^{\#c}$	0.0397
Week 8	162.85 (12.5)	162.85 (16.0)	148.57 (8.9) ^{a}	140.00 (8.1) $^{**\#\#b}$	0.0026
Week 9	152.85 (25.6)	151.42 (26.7)	150.00 (11.5) ^{b}	125.71 (15.1)	0.0687
P-value 2	0.0400	0.3322	0.0001	0.0001	

The *p-value* 1 indicates significance level for one-way ANOVA (followed by Tukey's post hoc) between group comparisons with *, $^{\#}$ and $^{\$}$ indicating significant levels with (*P<0.05, **P<0.01 and ***P<0.001) vs. G1, ($^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001) vs. G2 and $^{\$}$P<0.05 vs. G3. The *p-value* 2 indicates significance level for one-way ANOVA (followed by Tukey's post hoc) within the groups with alphabets indicating significant difference from week one, a=P<0.05, b=P<0.01 and c=P<0.001.

4.1.3 Fluid intake of the experimental groups

The weekly mean fluid intake was recorded and analyzed using one-way ANOVA followed by Tukey's post hoc test between and within group comparisons as shown in Table 3. There was significantly increased amount of fluid intake in the control (G1) group while decreased amount of fluid intake were recorded in the HFD only (G3), HFD treated with cocoa (G4) and the standard chow treated with cocoa (G2) groups in week one (1). Also, there were significantly decreased amount of fluid consumed in HFD only (G3) and HFD treated with cocoa (G4) when compared to both the control (G1) and the standard chow treated with cocoa (G2) in weeks two (2) and three (3). Week four (4) recorded significantly decreased amount of fluid intake in the HFD treated with cocoa (G4) group and increased amount of fluid intake in both the control (G1) and the standard chow treated with cocoa (G2) group. Similarly, there were significantly decreased amount of fluid intake in HFD only (G3) and increased amount of fluid intake in both the control (G1) and the standard chow treated with cocoa (G2) as well as decreased amount of fluid intake in the HFD treated with cocoa (G4) and increased amount of fluid intake in the control (G1) at week five (5).

This suggests that, the control (G1) and the standard chow treated with cocoa (G2) consumed more amount of fluid and the HFD only (G3) and the HFD treated with cocoa (G4) groups consumed less amount of fluid throughout the experimental period.

Table 3. Weekly mean fluid intake of rats in (milliliters) during the experimental period. Values are means with standard deviation in brackets**.**

Animal groups	G 1	G 2	G 3	G 4	P-value 1
Week 1	190.00 (15.0)	167.85 (12.1) *	150.00 (13.8) ***	155.00 (5.7) ***	0.0001
Week 2	174.28 (16.1)	175.71 (13.9)	150.00 (15.2) $^{*\#}$	144.28 (12.7) $^{**\#\#}$	0.0004
Week 3	170.00 (14.1)	171.42 (14.6)	150.00 (15.2) $^{*\#}$	144.28 (7.8) $^{**\#\#}$	0.0008
Week 4	167.14 (13.8)	167.14 (14.9)	147.14 (9.5)	138.57 (19.5) $^{**\#\#}$	0.0021
Week 5	171.42 (27.3)	180.00 (22.3)	148.57 (18.6)$^{*\#\#}$	151.42 (24.1)$^{\#}$	0.0499
Week 6	154. 28 (19.8) ^{a}	155.71 (16.1)	144.28 (12.7)	155.71 (16.1)	0.5121
Week 7	165.71 (17.1)	167.14 (17.9)	160.00 (18.2)	158.57 (15.7)	0.7454
Week 8	165.71 (13.9)	167.14 (13.8)	154.71 (30.4)	157.14 (11.1)	0.2007
Week 9	152.85 (24.3)	160.00 (32.6)	145.71 (30.4)	144.28 (28.7)	0.7337
P-value 2	0.0322	0.1171	0.9685	0.2693	

The *p-value* 1 indicates significance level for one-way ANOVA (followed by Tukey's post hoc) between group comparisons with *, and $^{\#}$ indicating significant levels with (*P<0.05, **P<0.01 and ***P<0.001) vs. G1, ($^{\#}$P<0.05 and $^{\#\#}$P<0.01) vs. G2. The *p-value* 2 indicates significance level for one-way ANOVA (followed by Tukey's post hoc) within the groups with a=P<0.05 indicating significant difference from week one.

4.2. Effect of HFD and natural cocoa on serum glucose levels

4.2.1 Effect of HFD and cocoa on fasting blood glucose (FBG) levels

Figure 12 represents assessment of weekly mean FBG in mmol/L between groups using one-way ANOVA followed by Tukey's post hoc test for the nine-week experimental period. There were statistically significant differences in weeks three (p= 0.0050), five (p = 0.0024), six (p= 0.0349), seven (p= 0.0009) and eight (p = 0.0126) between the various experimental groups. For week three (3), there was significantly decreased FBG levels observed in HFD treated with cocoa (G4) group when compared to HFD only (G3) group indicating decreased FBG levels in HFD treated with cocoa (G4) group while the HFD only (G3) group had increased FBG levels. For week five (5), there were significantly increased FBG levels recorded in HFD only (G3) group whereas both the control (G1) and the standard chow treated with cocoa (G4) groups had decreased FBG. Similarly, the sixth week recorded significantly decreased FBG levels in the HFD treated with cocoa (G4) group and increased FBG levels in HFD only (G3) group. Another significantly increased FBG levels was observed at week seven (7) between HFD only (G3) when compared to the HFD treated with cocoa (G4), control (G1) and standard chow treated with cocoa (G2) indicating increased FBG levels in HFD only (G3) group and decreased FBG levels in the HFD treated with cocoa (G4), control (G1) and the standard chow treated with cocoa (G2). Also, there was significantly increased FBG levels in the HFD only (G3) group whereas decreased FBG levels in the standard chow treated with cocoa (G2) group was observed in week eight (8).

From figure 12, it indicates that HFD increased blood glucose levels in rats in HFD only (G3) group when compared to other groups while rats in cocoa groups (G2 and G4) had decreased blood glucose levels. This suggests that HFD increased blood glucose levels and natural cocoa intake decreased blood glucose levels in HFD fed rats.

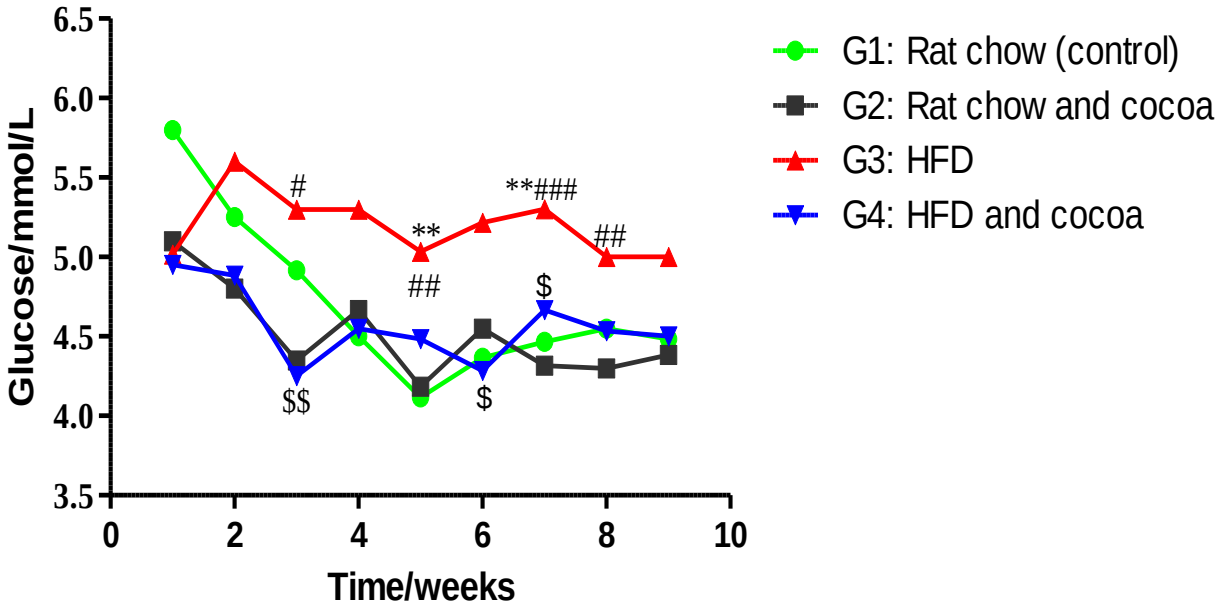


Figure 12. Line plot showing weekly mean FBG levels of rats during the experimental period. Each point represents mean glucose level of rats in the group for specific week. *P-value* represents significance level for one-way ANOVA (followed by Tukey's post hoc) for between-group comparison with *, $^{\#}$ and $^{\$}$ indicating level of statistically significant difference compared to G1, G2 and G3 respectively. **P<0.01, $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 and $^{\$}$P<0.05, $^{\$\$}$P<0.01.

4.2.2 Effect of HFD and cocoa on oral glucose tolerance test (OGTT) levels

Figure 13 represents assessment of mean OGTT and glucose levels after ingestion of bolus of glucose between groups using one-way ANOVA followed by Tukey's post hoc test for the various groups two days before the rats were sacrificed. There were statistically significant differences in glucose levels at time thirty (30 minutes) with p= 0.0060, ninety (90 minutes) with p= 0.0064 and one hundred and twenty (120 minutes) with p= 0.0001.

After the administration of the bolus of glucose, the glucose levels increased in all the animals with highest levels observed in the HFD treated with cocoa (G4) group when compared to the standard chow treated with cocoa (G2) and the HFD only (G3) groups at time point 30 minutes. There was also significantly increased glucose levels recorded in HFD only (G3) group whereas glucose levels decreased in the HFD group treated with cocoa (G4), standard chow treated with cocoa (G2) and the control (G1) groups recorded at time point 90 minutes. Finally, statistically significant differences in glucose levels were observed at time point 120 minutes between HFD only (G3) group when compared to the control (G1), standard chow treated with cocoa (G2) and HFD treated with cocoa (G4) indicating increased glucose levels in HFD only (G3) group whereas decreased glucose levels recorded in HFD treated with cocoa (G4), standard chow treated with cocoa (G2) and the control (G1) groups. The glucose levels of HFD treated with cocoa (G4), standard chow treated cocoa (G2) and the control (G1) decreased significantly and returned to the baseline at time point 120 minutes whereas that of the HFD only (G3) group almost maintained high glucose levels after it shot up within 30 minutes.

From figure 13, it indicates that HFD impaired glucose homeostasis in rats in HFD only (G3) group when compared to other groups while rats in cocoa groups (G2 and G4) had improved glucose homeostasis. This suggests that HFD impaired glucose homeostasis and natural cocoa intake improved glucose homeostasis in HFD fed rats.

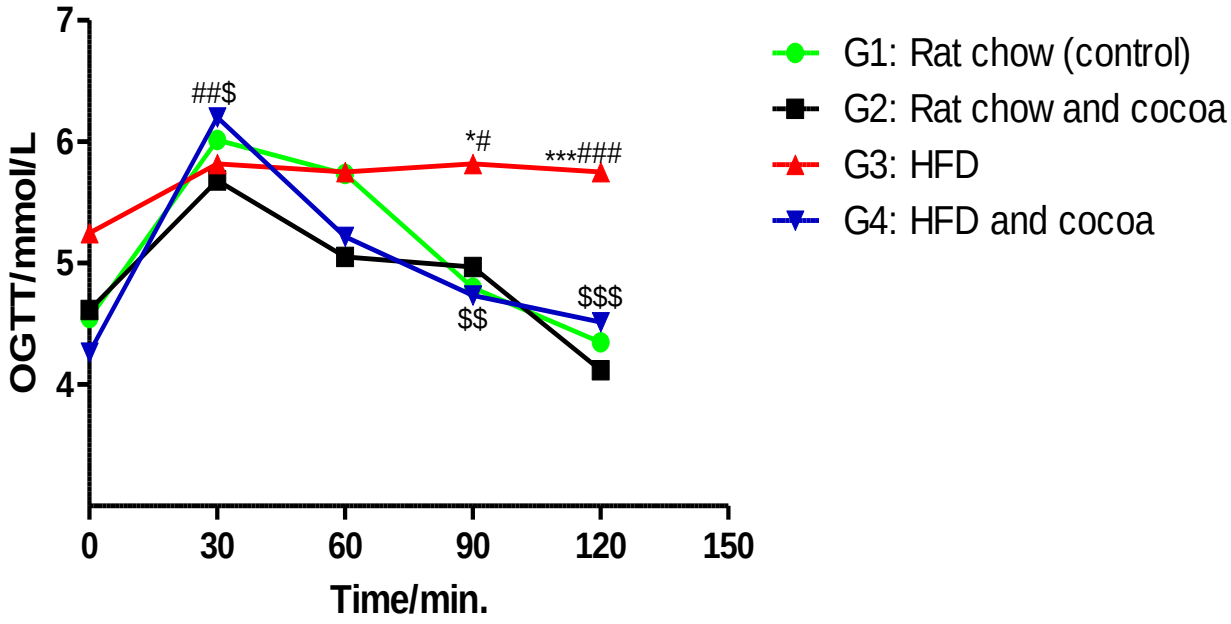


Figure 13. Line plot showing mean OGTT levels of rats two days before experimental period. Each point represents mean glucose level of rats in the group for specific time. *P-value* represents significance level for one-way ANOVA (followed by Tukey's post hoc) for between-group comparison with *, $^{\#}$ and $^{\$}$ indicating level of statistically significant difference compared to G1, G2 and G3 respectively. $^{*}P<0.05$, $^{***}P<0.001$, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, $^{\$}P<0.05$, $^{\$\$}P<0.01$ and $^{\$\$\$}P<0.001$

4.3 Effect of HFD and natural cocoa on weight of rat livers

Figure 14 shows a graph of the mean gross weight of rat liver harvested at the end of the experiment. A one-way ANOVA followed by Tukey's post hoc test showed a significant difference (p= 0.0034) between HFD only (G3) group when compared to both the control (G1) and the standard chow treated with cocoa (G2) groups. This indicates that there was increased gross weight of rat liver in HFD only (G3) group whereas decreased gross weight of rat liver

observed in both the control (G1) and the standard chow treated with cocoa (G2) groups. There was also increased gross weight of rat liver in HFD only (G3) when compared to HFD treated with cocoa (G4) group but not statistically significant.

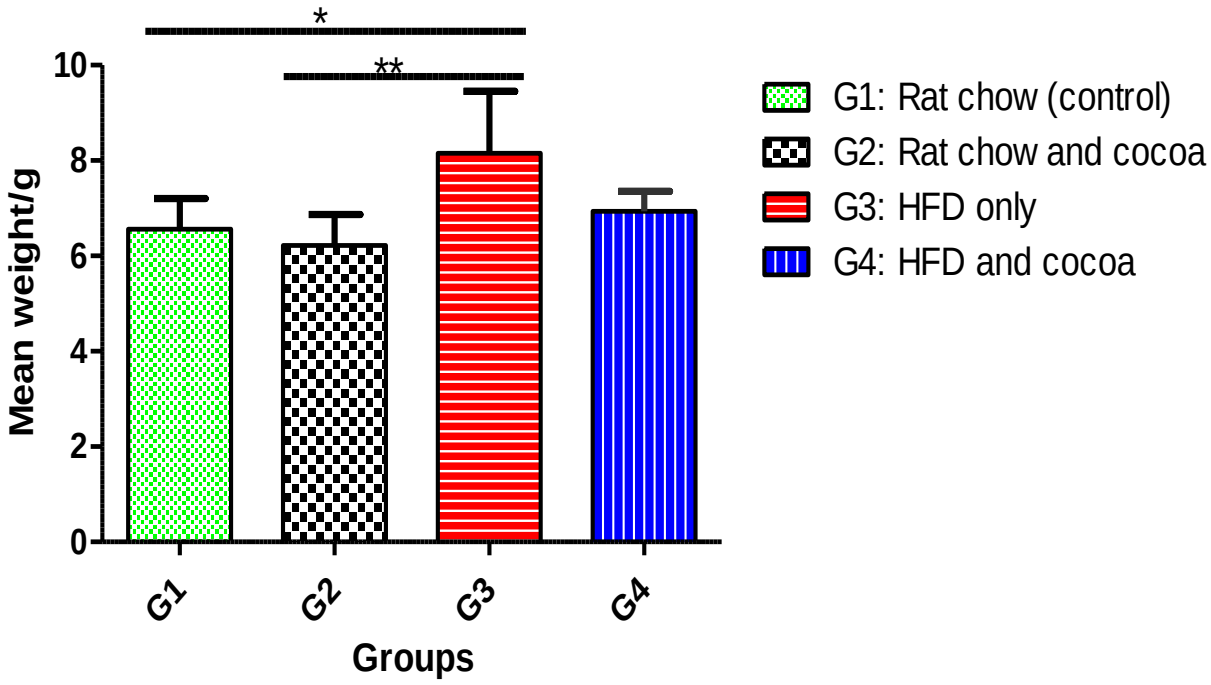


Figure 14. Bar chart showing mean liver weight of the rats in the various groups after the termination of the experiment (9 weeks) of high-fat diet feeding. Each column represents mean with SD as error bars indicates the significant level for one-way ANOVA (followed by Tukey's post hoc test) for between group comparisons. ($^{*}P<0.05$) vs. G1 and (**$P<0.01$) vs. G2

4.4 Histological and morphometric analysis of adipose tissues and liver cells

4.4.1 Effect of HFD and natural cocoa on adipocyte size.

After the treatment of HFD and natural cocoa, the adipocyte sizes increased in HFD only group and decreased in cocoa treated groups. The assessment was performed by two observers (observer

1 and observer 2) to eliminate biasness of result. Observer 2 had no prior knowledge of the treatment groups. The results obtained by the two observers were compared and it was found out that there was no significant differences between the results of observer 1 and observer 2. The results of the two observers are shown below but only one was discussed in detail.

4.4.2 Effect of HFD and natural cocoa on epididymal adipocyte

Figures 15 and 16 show the mean epididymal adipocyte sizes of rats in the various groups. One-way ANOVA followed by Tukey's post hoc test showed statistically significant difference ($p<0.0019$) in epididymal adipocyte size between HFD only (G3) group when compared to the HFD treated with cocoa (G4), the control (G1), and the standard chow treated with cocoa (G2) groups indicating that the HFD only group (G3) had increased epididymal adipocyte size while HFD treated with cocoa (G4), control (G1) and the standard chow treated with cocoa (G2) had decreased epididymal adipocyte size both in the results obtained by observer 1 and observer 2. The *p-value* for observer 1 was ($p<0.0019$) and that of observer 2 was ($p<0.0001$).

From figures 15 and 16, it indicates that HFD increased epididymal adipocyte size in the HFD only group (G3) when compared to all other groups and natural cocoa decreased epididymal adipocyte size.

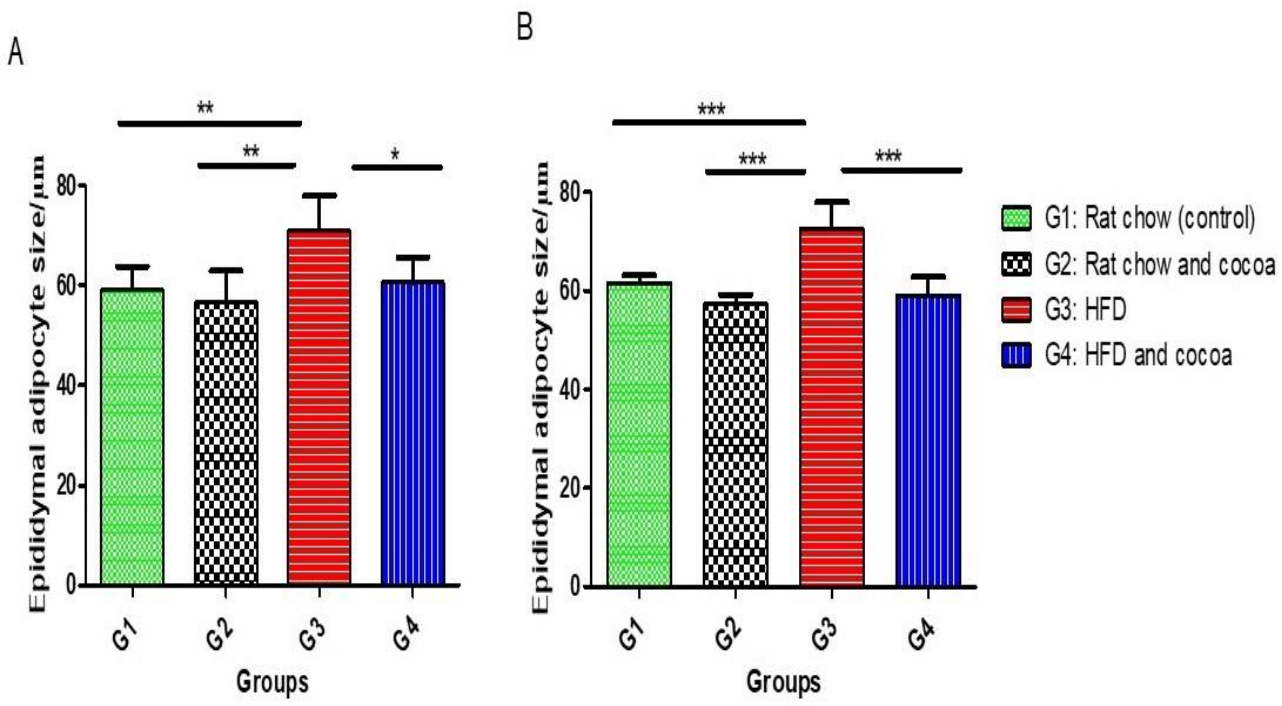


Figure 15. Bar chart showing mean epididymal adipocyte size by two (2) observers (A is observer 1 and B is observer 2) of the various experimental rats after high-fat diet feeding. Each column represents mean with SD as error bars. The *p-value* indicates the significant level for one-way ANOVA (followed by Tukey's post hoc test) for between group comparisons. *P<0.05, **P<0.01 and ***P<0.001.

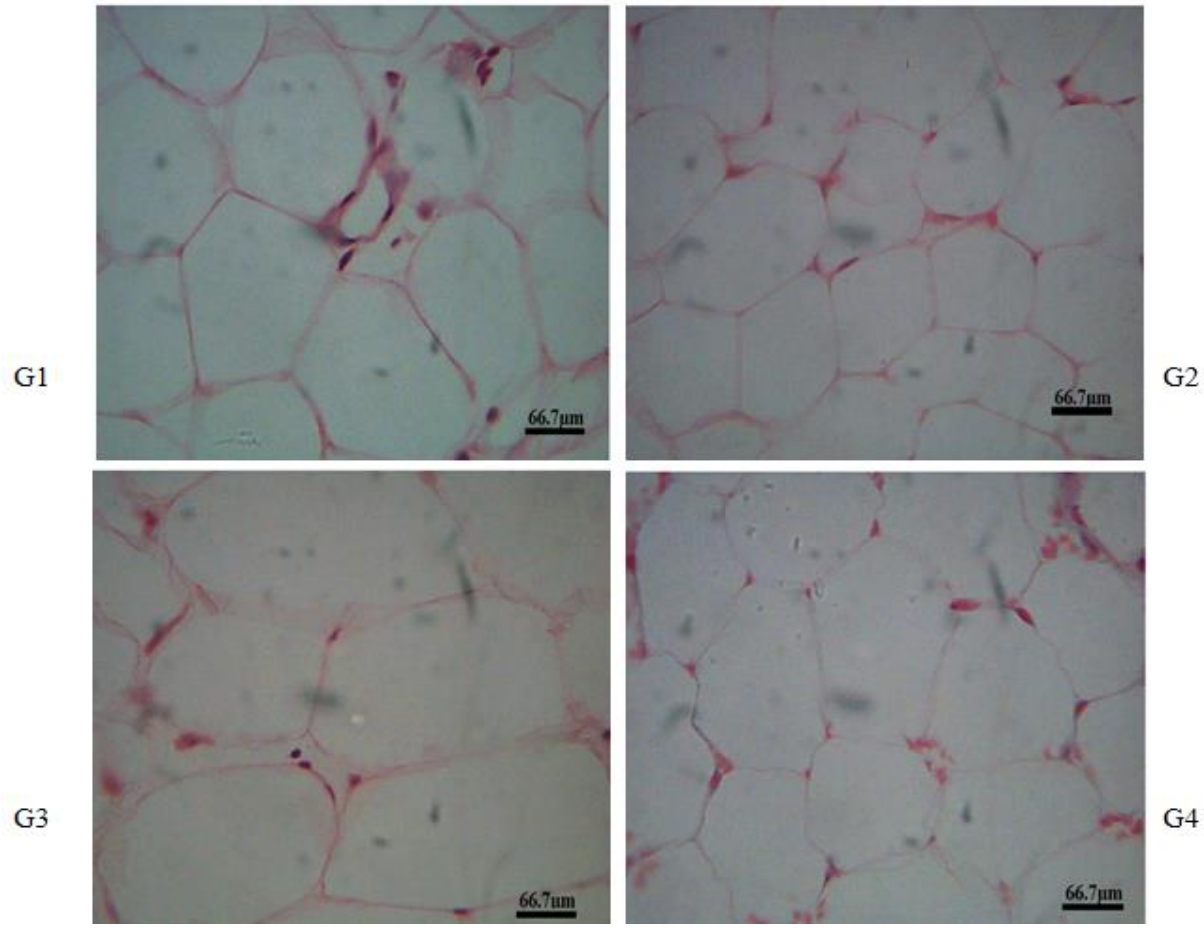


Figure 16. Photomicrographs of Haematoxylin and Eosin (H&E) stained sections of epididymal adipose tissues from the four groups of rats studied. G1: Standard chow and tap water (control), G2: Standard chow treated with cocoa, G3: HFD only and G4: HFD treated with cocoa.

4.4.3 Effect of HFD and cocoa on intestinal adipocyte size

The mean intestinal adipocyte size of rats in the various groups are shown in figures 17 and 18. One-way ANOVA followed by Tukey's post hoc test showed statistically significant differences ($p<0.0002$) in intestinal adipocyte sizes between HFD only (G3) group when compared to the HFD treated with cocoa (G4), the control (G1), and the standard chow treated with cocoa (G2) groups indicating that the HFD only group (G3) had increased intestinal adipocyte sizes whereas

HFD treated with cocoa (G4), control (G1) and the standard chow treated with cocoa (G2) groups had decreased intestinal adipocyte sizes in the results obtained by both observer 1 and observer 2. The *p-value* for observer 1 was ($p<0.0002$) and that of observer 2 was ($p<0.0015$).

From figures 17 and 18, it indicates that HFD increased intestinal adipocyte sizes in the HFD only group (G3) when compared to all the groups. This suggests that HFD increased intestinal adipocyte sizes and natural cocoa decreased intestinal adipocyte sizes.

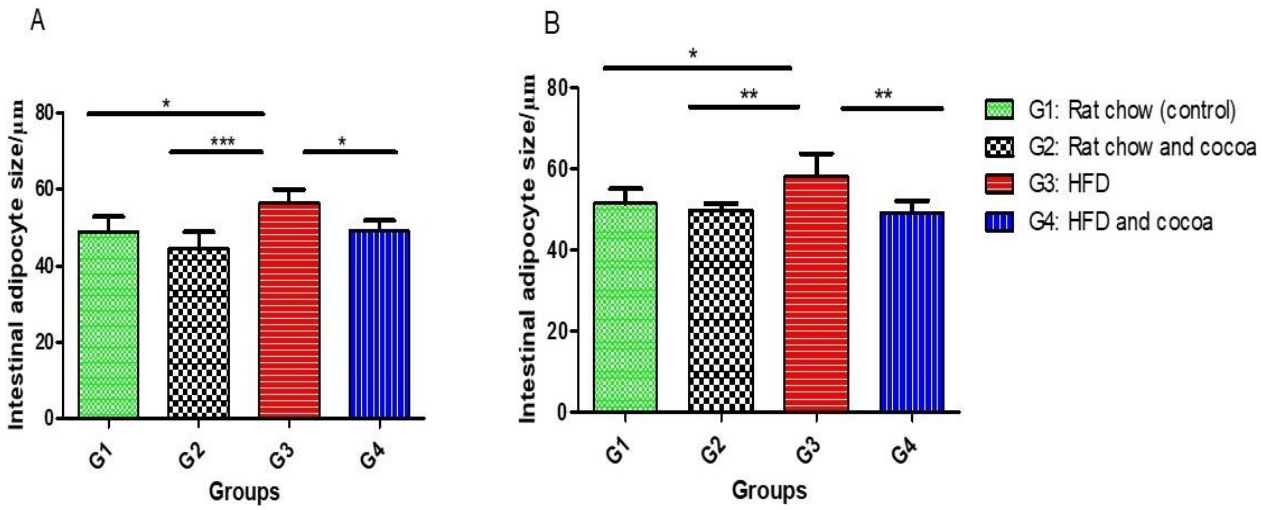


Figure 17. Bar chart showing mean intestinal adipocyte size by two (2) observers (A is observer 1 and B is observer 2) of the various experimental rats after the experiment of high-fat diet feeding. Each column represents mean with SD as error bars. The *p-value* indicates the significant level for one-way ANOVA (followed by Tukey's post hoc test) for between group comparisons. *P<0.05, **P<0.01 and ***P<0.001.

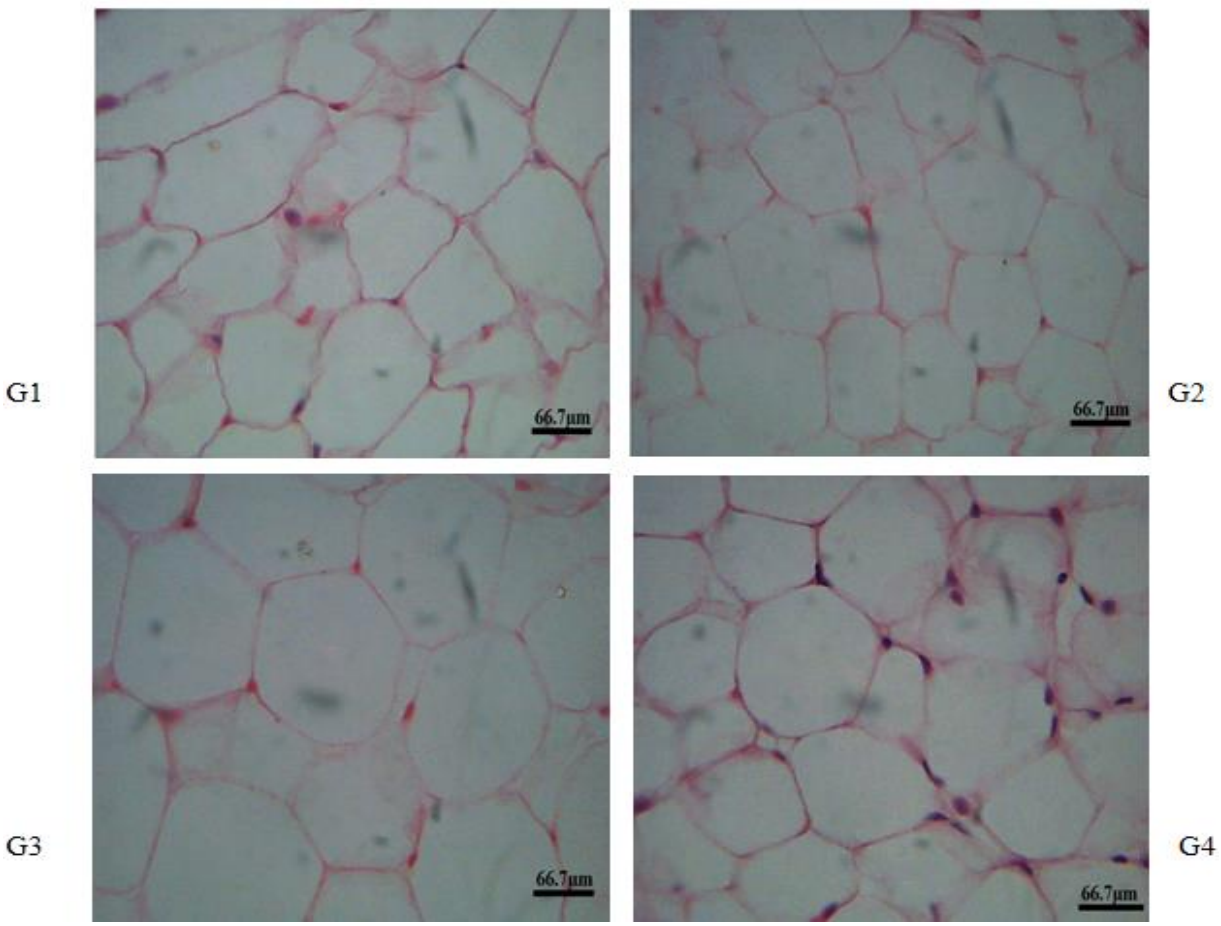


Figure 18. Photomicrographs of Haematoxylin and Eosin (H&E) stained sections of intestinal adipose tissue from the four groups of rats studied. G1: Standard chow and tap water (control), G2: Standard chow treated with cocoa, G3: HFD only and G4: HFD treated with cocoa

4.4.4 Effect of HFD and cocoa on mesenteric adipocyte size

Figures 19 and 20 show the mean mesenteric adipocyte size of rats in the various groups after the treatment. One-way ANOVA followed by Tukey's post hoc test showed statistically significant differences ($p<0.0008$) in mesenteric adipocyte sizes between HFD only (G3) group when compared to the HFD treated with cocoa (G4), the control (G1), and the standard chow treated with cocoa (G2) group indicating that the HFD only group (G3) had increased mesenteric adipocyte sizes whereas HFD treated with cocoa (G4), control (G1) and the standard chow treated

with cocoa (G2) had decreased mesenteric adipocyte sizes in the results obtained by both observer 1 and observer 2. The *p-value* for observer 1 was ($p<0.0008$) and that of observer 2 was ($p<0.0008$).

From figures 19 and 20, it indicates that HFD increased mesenteric adipocyte sizes in the HFD only group (G3) when compared to all the groups. This suggests that HFD increased mesenteric adipocyte sizes and natural cocoa decreased mesenteric adipocyte sizes.

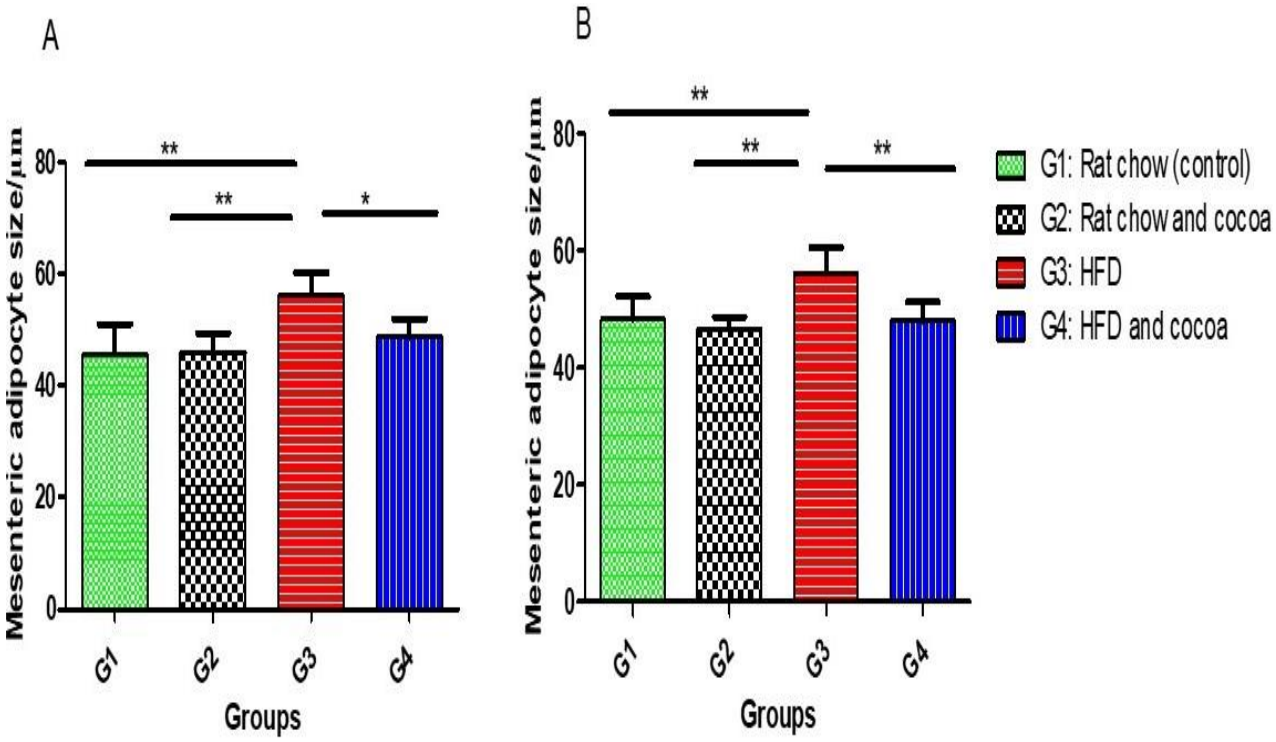


Figure 19. Bar chart showing mean mesenteric adipocyte size by two (2) observer (A is observer 1 and B is observer 2) of the various experimental rats after the experimental period of high-fat diet feeding. Each column represents mean with SD as error bars. The *p-value* indicates the significant level for one-way ANOVA (followed by Tukey's post hoc test) for between group comparisons. *P<0.05 and **P<0.01.

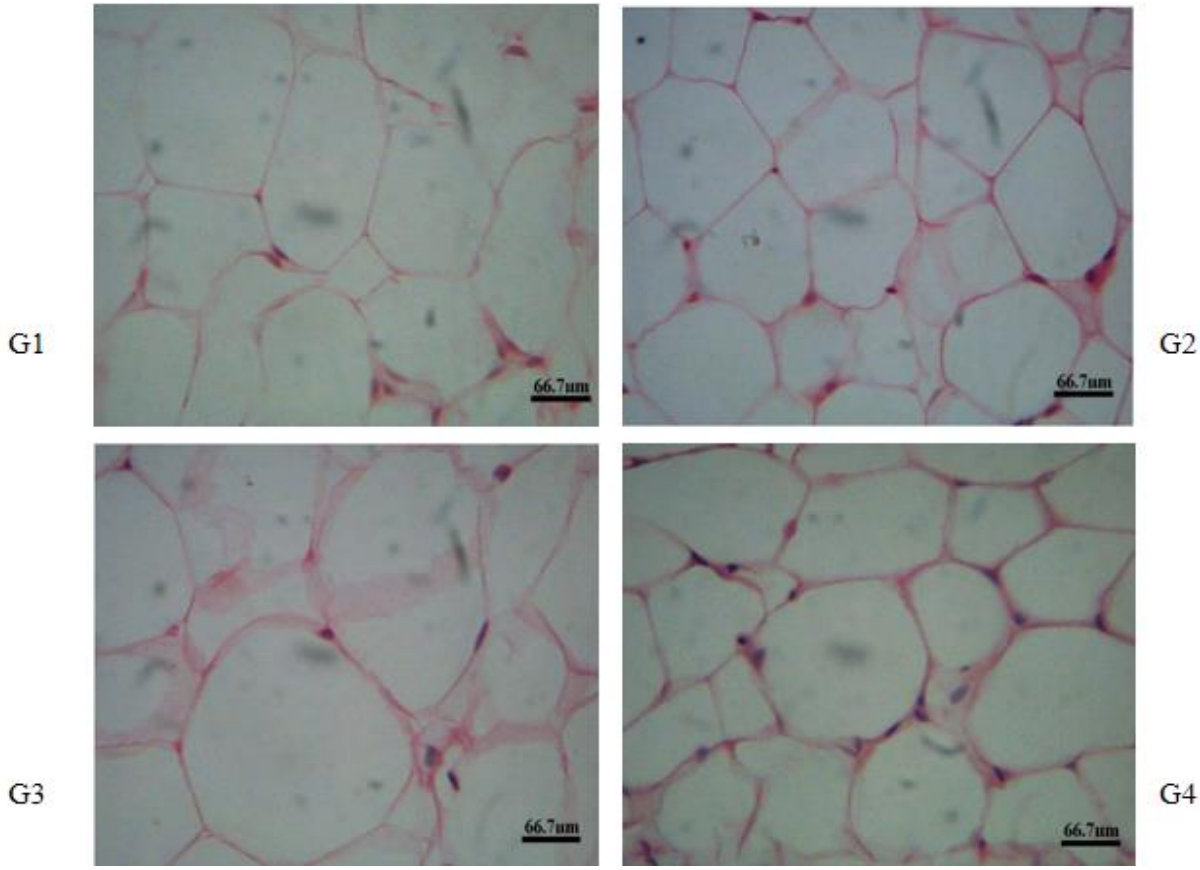


Figure 20. Photomicrographs of Haematoxylin and Eosin (H&E) stained sections of mesenteric adipose tissue from the four groups of rats studied. G1: Standard chow and tap water (control), G2: Standard chow treated with cocoa, G3: HFD only and G4: HFD treated with cocoa

4.4.5 Effect of HFD and natural cocoa on the liver hepatocytes

Figures 21 and 22 show the mean volume density of fat droplet infiltration in the hepatocytes of rats in the various experimental groups. One-way ANOVA followed by Tukey's post hoc test showed statistically significant differences ($p<0.0011$) in volume densities of fat droplets in the hepatocytes of rats between HFD only (G3) group when compared to the HFD treated with cocoa (G4), the control (G1), and the standard chow treated with cocoa (G2) indicating that the HFD

only group (G3) had increased volume density of fat infiltration in the hepatocyte of rat whereas HFD treated with cocoa (G4), control (G1) and the standard chow treated with cocoa (G2) had decreased volume density of fat infiltration in the hepatocytes of rats.

From figures 21 and 22, it indicates that HFD increased fat infiltration in the hepatocytes of rats in the HFD only group (G3) when compared to all the groups. This suggests that HFD increased fat infiltration in the hepatocytes of rats and natural cocoa decreased fat infiltration in the hepatocytes of rats.

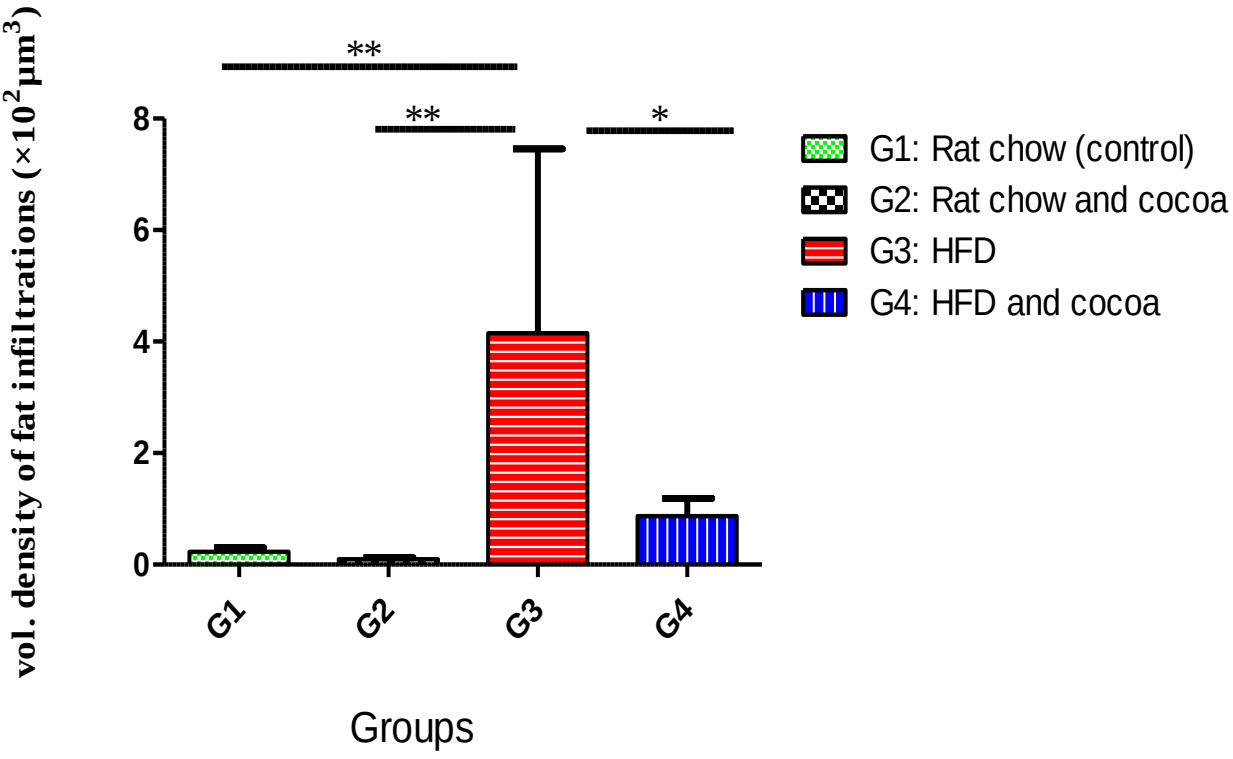


Figure 21. Bar chart showing mean volume density of fat infiltration in the hepatocytes from four groups of rats studied. Each column represents mean with SD as error bars. The *p-value* indicates the significant level for one-way ANOVA (followed by Tukey's post hoc test) for between group comparisons. *P<0.05, **P<0.01.

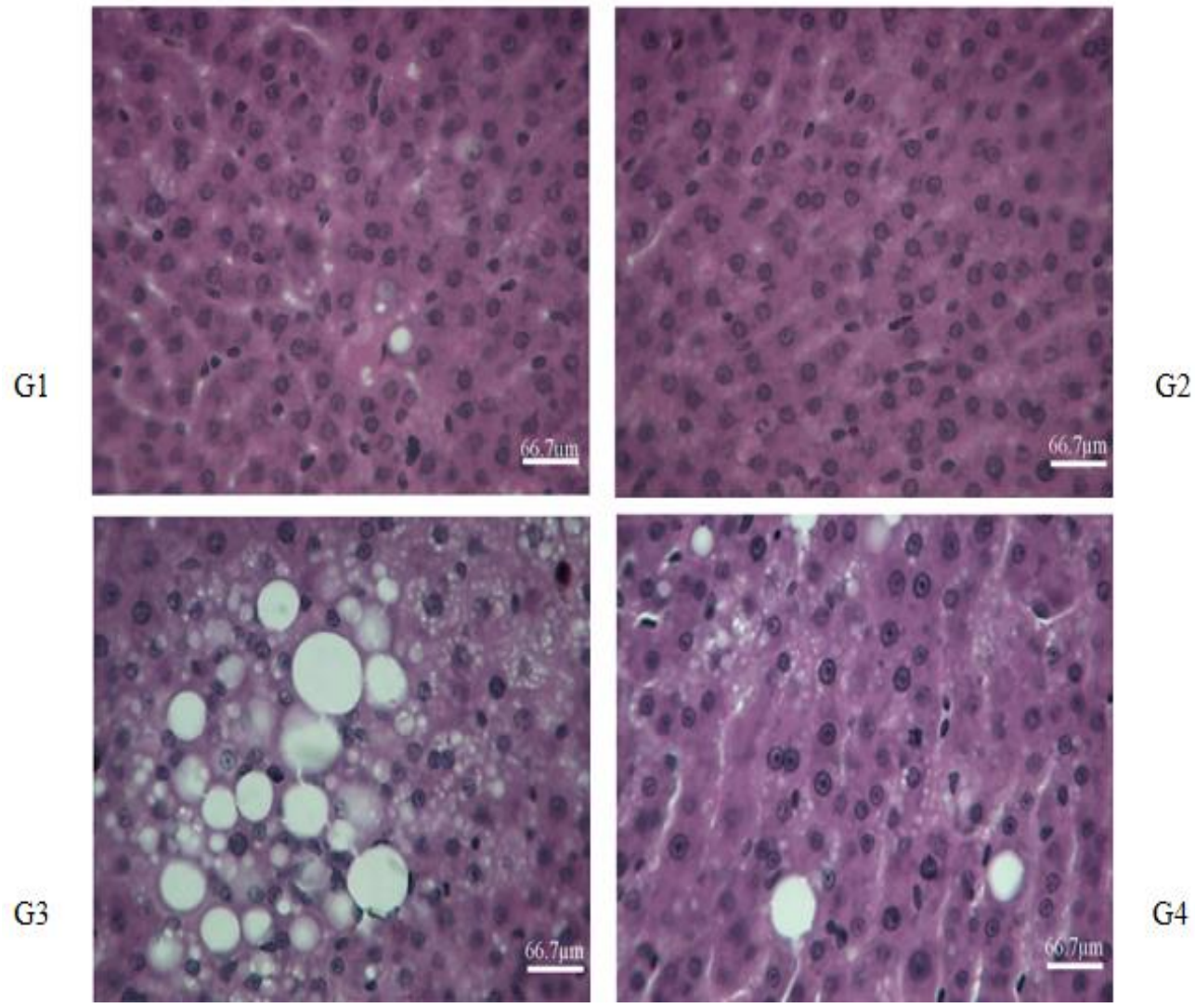


Figure 22. Photomicrographs of Haematoxylin and Eosin (H&E) stained sections of hepatocytes from the four groups of rats studied. G1: Standard chow and tap water (control), G2: Standard chow treated with cocoa, G3: HFD only and G4: HFD treated with cocoa.

4.5 Effect of HFD on tumor necrosis factor-alpha (TNF-α), an inflammatory biomarker

The mean concentration of TNF-α of rats for pretreatment (A) and post-treatment (B) of the various groups are shown in Figure 23. One-way ANOVA followed by Tukey's post hoc test showed no

statistically significant difference ($p<0.0237$) for pretreatment and ($p<0.2575$) for post-treatment in the concentration of TNF-α between all the groups. However, the concentration of TNF-α in all the groups decreased with HFD only (G3) having a slightly higher concentration in the serum of post treatment but not statistically significant compared to other groups.

This suggests that there were low concentrations of TNF-α in the serum of the experimental rats in all the groups at end of the treatment.

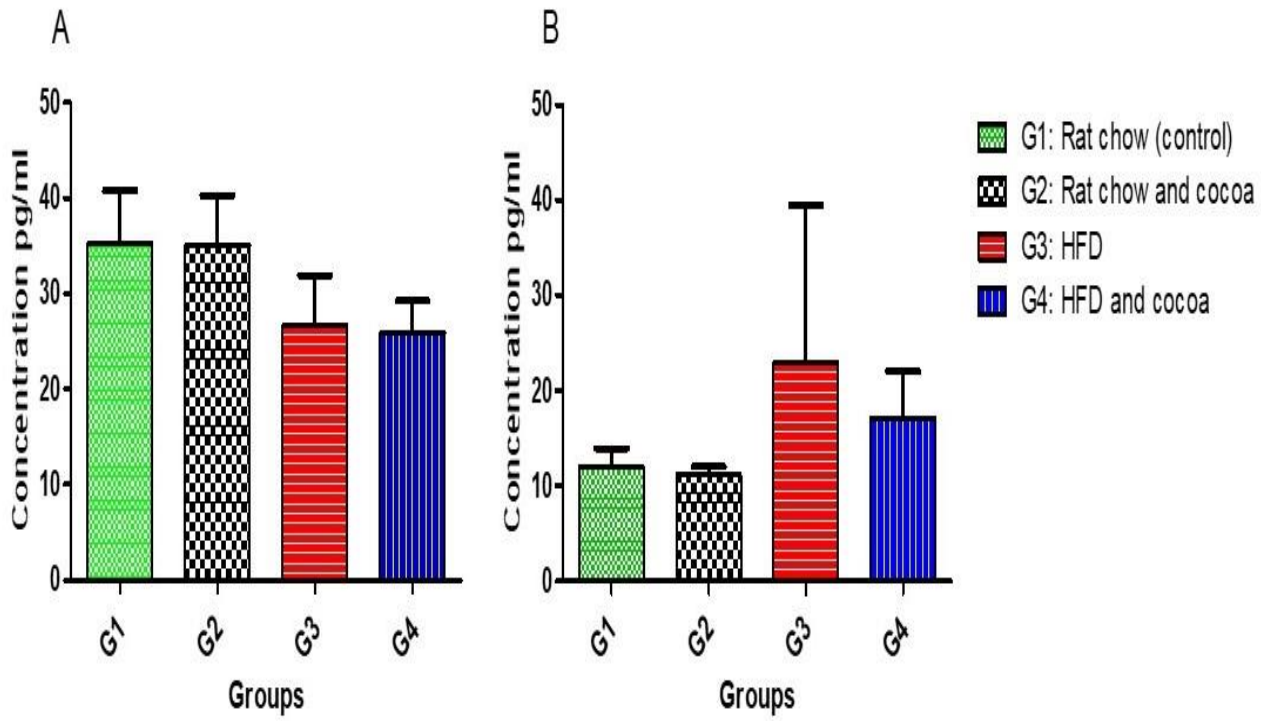


Figure 23. Bar chart showing mean concentration levels of TNF-α of rats for pretreatment (A) and post treatment (B) from the four (4) groups of rats studied. Each column represents mean with SD as error bars. The *p-value* indicates the significant level for one-way ANOVA between group comparisons.

Table 4: Summary of statistics on concentration of TNF-α for pretreatment and post treatment in the various experimental groups

Inflammatory marker	G1	G2	G3	G4	P-value
Pretreatment TNF-α concentration	35.27 (SD 5.5)	35.04 (SD 5.2)	26.77 (SD 5.1)	25.92 (SD 3.3)	0.0237
Post-treatment TNF-α concentration	12.07 (SD 1.8)	11.15 (SD 0.8)	23.02 (SD 16.4)	17.13 (SD 4.9)	0.2575

Values are expressed as means (SD). The *p-values* indicates significance level for one-way ANOVA (followed by Tukey's post hoc) between group comparisons

CHAPTER FIVE

5.0 DISCUSSION

5.1 Effects of HFD and cocoa on weight gain and obesity

The weight gain in rats treated with HFD only increased due to chronic intake of HFD whereas natural cocoa intake decreased weight gain. The increased weight gain observed in HFD only group could be ascribed to the intake of HFD as several studies have shown that HFD increases fat mass and adiposity with subsequent weight gain in an individual (Preguiça *et al*., 2020; Stolarczyk, 2017; Gao *et al*., 2015). Although, HFD only group consumed less amount of feed, chronic intake of HFD increased weight gain of the animal (Tan, 2019). The kind of saturated fatty acids found in coconut oil are poorly utilized for energy (Lima & Block, 2019) and rather continues to be acylated into triglyceride and stored in the adipose tissue and this may have contributed to the increased weight gain (Dinicolantonio, 2017).

In contrast, natural cocoa decreased weight gain in rats that fed on HFD treated with cocoa as well as rats fed on standard chow and natural cocoa. The decreased weight gain could be attributed to the natural cocoa as an intervention in this group. According to reports, cocoa intake enhances metabolism of fat and thus reducing storage of fat in adipose tissues with subsequent decrease in weight gain (Coronado-Cáceres *et al*., 2019). Other reports also show that cocoa polyphenols have anti-obesity effect due to their capacity to suppress fatty acid synthesis and stimulate energy expenditure in the mitochondria thereby reducing weight gain (Andújar *et al*, 2012).

5.2 Role of HFD and cocoa intake on glucose homeostasis

Results from the study showed that the mean fasting blood glucose (FBG) levels increased in the high-fat diet only group (G3), but decreased in the HFD treated with natural cocoa group as well as standard chow treated with natural cocoa (G2). Also, oral glucose tolerance test (OGTT) results suggests that HFD reduces post-prandial tolerance of glucose, whereas intake of natural cocoa improves glucose tolerance. The increased FBG and decreased glucose tolerance levels in HFD only group may be associated with HFD which enhanced adipocyte expansion and dysfunction resulting in accumulation of fat droplets in the hepatocytes with subsequent impairing of insulin signaling to act on glucose levels thereby increasing blood glucose levels. Reports show that upon HFD consumption, the storage capacity of the adipose tissue is exceeded in obese state and free fatty acids begin to accumulate in metabolic tissues such as liver, skeletal muscle, heart and pancreas resulting in insulin resistance (Longo *et al*., 2019; van Herpen & Schrauwen-Hinderling, 2008). According to Perona, (2017), high fatty acids in diet affect the configuration of cell membranes which influence the affinity of receptors for insulin and its action on glucose thereby increasing glucose levels in cells. Epidemiological studies have also shown that insulin sensitivity is impaired when the total energy intake is about 35-40% (Parillo & Riccardi, 2004).

Similarly, previous studies show that high-fat diet triggers elevated insulin levels and insulin resistance affecting blood glucose-lowering effect of insulin in animal studies (Woods *et al*., 2018; Czech & Building, 2018; Parillo & Riccardi, 2004) and reports by Abdul-Ghani & Defronzo, (2010) and Parillo & Riccardi, (2004) suggested that when the fat intake makes up 40% of diet, abnormalities such as reduction in insulin receptors, metabolism and glucose transport as well as decline in liver and synthase activity in muscle glycogen develop, inhibiting insulin action on glucose. Moreover, increased glucose levels accompanied by reduced glucose tolerance could be

due to High-fat diet which results in the accumulation of reactive oxygen species which induces the development of insulin resistance in metabolic tissues such as adipose tissues and peripheral tissues promoting high blood glucose levels (Flores, 2019).

The decreased levels of FBG and improved glucose tolerance in rats treated with HFD and cocoa as well as the standard chow and cocoa groups may possibly be attributed to the presence of flavonoids, polyphenols, methylxanthine and theobromine capable of improving the homeostasis of glucose and insulin levels. Cocoa polyphenols activate nuclear factor erythroid 2 (Nrf2) which induces the transcription of antioxidant enzymes blocking the production of ROS, reducing oxidative stress and insulin resistance (Flores, 2019). According to studies the polyphenolic-rich cocoa extract contain hypoglycemic effect reducing glucose levels in blood (Ruzaidi *et al*., 2008). Cocoa polyphenols activate hepatic glycogen synthase by boosting the expression of Phosphatidylinositol 3-Kinase (PI3K/AKT) and Glycogen Synthase Kinase 3 (GSK3) in hepatic cells and liver of insulin resistant rats which enhances glucose homeostasis (Martín *et al.*, 2014). Cocoa polyphenols also prevent rise of Phosphoenolpyruvate Carboxykinase (PEPCK) levels which are known to activate gluconeogenesis (Brand-miller *et al*, 2007). Studies conducted by Rowley (2017); Medicine *et al*. (2012); Martín *et al*. (2010) suggest that polyphenols and methylxanthine in cocoa contribute to improve insulin sensitivity and decrease both fasting and postprandial glucose levels in blood. Oligometric, procyanidins and epicatechin monomers in cocoa have the capacity in maintaining fasting glucose, glucose tolerance, improving insulin sensitivity, normalize blood glucose levels and stimulate the pancreatic β cells to secrete insulin and respond more efficiently to increased blood glucose (Dorenkott *et al.*, 2014). Epicatechin monomers found in cocoa stimulate the release of insulin to act on the increased blood glucose levels induced by high-fat diet (Rowley *et al*., 2017).

5.3 Morphological changes in adipocyte and hepatocyte of rats treated with high-fat diet

Histological assessment performed in this study revealed observable morphological changes in the adipose tissue microarchitecture of all the groups but to a greater extent in the high-fat diet only group. However, there were enhancement in the microarchitecture of the adipocytes and hepatocytes in the animals treated with high-fat diet and cocoa as well as standard chow and cocoa fed groups.

5.3.1 Role of HFD and cocoa on morphological changes in adipocyte (hypertrophy)

High-fat diet only increased adipocyte sizes (hypertrophy) while in the HFD treated with natural cocoa and standard chow treated with natural cocoa groups had decreased adipocyte sizes. The highest mean epididymal adipocyte diameter recorded was 71.06µm in HFD only and smallest of 56.15µm recorded in the standard chow and cocoa group as shown in table 8. The highest mean intestinal adipocyte diameter recorded was 56.36µm in HFD only and smallest was 44.57µm in standard chow and cocoa group as shown in table 9. The widest mesenteric adipocyte diameter recorded was 56.17µm in HFD and tap water group and smallest 45.59µm in standard chow and cocoa group as shown in table 10.

The increased adipocyte sizes could be ascribed to the high-fat diet (40% of fat) which is consistent with increased weight gain. As animals consume more fat, adipocyte sizes increase leading to increased adiposity and weight gain or obesity. Research shows that, rats on HFD progressively gain more fat mass and subsequently display accelerated body weight gain which is accompanied by adipocyte hypertrophy compared to rats on standard chow (Gao *et al.*, 2015). Findings by Choe *et al.*, (2016) also suggests that, administration of HFD causes deleterious alterations to the morphology of the adipocytes, pancreatic islets which is believed to have effects on metabolic properties such as insulin resistance and adipokine secretion. Another report by Tan, (2019) shows

that chronic ingestion of fat results in a net energy overload with subsequent expansion of adipocytes. Stolarczyk, (2017), hypothesized that upon HFD feeding, adipose cells enlarge and increase in size in overweight and obese individuals creating an inflammatory setting. Another report suggests that sustained or chronic fat consumption contributes to adipose tissue hypertrophy resulting in dysfunctional adipocytes and metabolic inflammation (Tan, 2019).

It was shown that epididymal adipocytes had the widest diameter compared to intestinal and mesenteric adipose tissue, suggesting that epididymal adipocytes were more susceptible to become hypertrophic upon HFD intake. This finding is consistent with an earlier report suggesting that epididymal adipocytes diameter increased in both animals feeding on low and high-fat diets but to a greater extent in high-fat diet individuals (Poret *et al*., 2018). It can be suggested that epididymal adipocytes are more prone to inflammation since enlarged adipocytes are correlated to obesity and modulator for the secretion of adipokine.

The control and the standard chow treated with cocoa groups consumed high amount of feed than the HFD fed groups. However, the mean weight gain in HFD only increased. Studies show that, there is an increased accumulation of body fat and increased body weight gain on ingesting moderate diet or high diet containing saturated fatty acid (Aller *et al*., 2011; Coelho *et al.*, 2011; Bray & Popkin, 1998). Reports by Buettner, (2007) and Timmers *et al.*, (2010) revealed that prolonged high-fat diets intake trigger weight gain in rats. It is further proposed by Coelho *et al*., (2011) that rats exposed to a high saturated fat diet put on greater adiposity leading to hypertrophic adipocytes.

The decreased adipocyte sizes in high fat diet treated with natural cocoa could be due to the protective effect of natural cocoa. Regular natural cocoa intake reduced weight gain and adipocyte

sizes thus, improving adipocyte morphology (Martin *et al*., 2010). The decrease in the adipocyte sizes in the cocoa group is consistent with a report by Coronado-Cáceres *et al*., (2019) and Jang *et al*., (2015), who showed that theobromine in cocoa decreased the mass of mesenteric and epididymal fat in HFD treated animals resulting in decreased adipocyte sizes and reduced damages to adipocyte morphology. Theobromine inhibits adipocyte differentiation through a signal transduction pathway involving Adenosine Monophosphate activated Protein Kinase (AMPK) activation and inhibition of External Signal-Regulated Kinase (ERK) and c-Jun N-terminal Kinase (JNK) pathways (Jang *et al*., 2015).

5.3.2 Morphological changes of the hepatocytes (fat infiltration)

Histological evaluation of the liver sections revealed observable alterations in the architecture of the liver in the high-fat diet only group. Stained sections showed increased presence of fat droplets infiltrating in the hepatocytes (liver steatosis) whereas on the other hand there was improvement of hepatocyte morphology in the groups ingesting cocoa as intervention.

High-fat diet resulted in increased fat infiltration in the hepatocytes and consumption of natural cocoa resulted in reduced fat infiltration in the hepatocytes. The increased fat infiltration in the hepatocytes could be due to nutrient overload resulting in reduced capacity of adipose tissue to store the excess energy or fat leading to accumulation of fat droplets in the hepatocytes. Several studies in mice, rats and humans have shown that diets containing high amount of fat rapidly enhance hepatic steatosis while low fat diets decrease liver fat content (De Meijer et al., 2010; Jensen *et al*., 2010)). The results of this study is consistent with a study by Buettner, (2007) who showed that high-fat diets trigger hepatic steatosis and hepatic insulin resistance. Also, regular HFD consumption enhance fat infiltration in the hepatocytes since the liver is the organ that metabolizes and processes fats (Osna *et al*., 2017).

It was revealed from the study that increased adipocyte sizes and increased liver weight coincided with increased fat infiltration in the hepatocytes of rats in the HFD only suggesting that enlarged adipocytes enhance or maybe associated with the deposition of fat droplets in the hepatocytes impairing their normal functioning. Intake of HFD put stress on the adipose tissue failing to store the excess fatty acids depositing them into metabolic tissues including the liver (Lumeng & Saltiel, 2011)

However, the amount of fat droplets infiltrating the liver and mean liver weight in in the cocoa fed rats group significantly reduced, indicating that cocoa may exert protective effect on liver hepatocytes against the development of steatosis. This is consistent with a study conducted by Arora *et al*., (2015) whose findings revealed that consumption of cocoa polyphenols mop up free fatty acid in the liver and other tissues. Cocoa also suppresses the synthesis system of fatty acids in the liver reducing fatty acid accumulation (Andújar *et al*, 2012; Matsui *et al*., 2005).

5.4 Effect of HFD and cocoa on inflammation and TNF-α concentration in the blood serum.

The concentration of TNF-α was decreased in all the treatment groups after 9 weeks. The results showed that, as adipocytes expand due to nutrient overload, TNF-α levels decreased. This is in contrast with studies where inflammatory adipokine levels increased as adipocyte sizes expand for 12 weeks of HFD feeding (Fuster *et al*., 2016; Gao *et al*., 2015; Unger & Scherer, 2010). Several studies show that obesity initiates the release of inflammatory cytokines (Fuster *et al*., 2016; Gao *et al*., 2015; Unger & Scherer, 2010) suggesting that obesity initiates systemic inflammation. In this study, weight gain of the animals (rats) may not have been high enough to initiate inflammation and the release of TNF-α.

HFD treatment was expected to increase inflammatory markers. The contrary results maybe due to several reasons. Firstly, the experimental period of 9 weeks was too short to induce obesity in the animals. In contrast to this study, an existing data show that TNF-α levels were significantly elevated in HFD mice at 40 weeks (van der Heijden *et al*., 2015), suggesting that systemic inflammation maybe secondary to obesity and a longer experimental period would have been ideal. Moreover, other adipokines such as IL-1β, IL-1α and IL-6 may be released within the experimental period other than TNF-α as indicated by (Poret *et al*., 2018). Although the expression of TNF-α was low in the serum, it is possible the biomarker could be expressed in the adipose and the liver tissues rather than serum as other reports show that TNF-α was expressed in the tissues of rats at the end of 7 weeks after a HFD intake (Guillemot-Legris *et al*., 2016; Poret *et al*., 2018).

5.5 Summary of key findings

- High-fat diet increased weight gain significantly and increased fasting blood glucose levels and the intake of natural cocoa reduced weight gain and fasting blood glucose levels.
- High-fat diet intake impaired glucose tolerance whereas natural cocoa improved post-prandial glucose tolerance in rats.
- The intake of high-fat diet resulted in hypertrophy and the alterations in the morphology of adipocytes and this deleterious effect was reduced by the regular intake of cocoa.
- High-fat diet increased infiltration of fat droplets in the hepatocytes (liver steatosis) altering liver architecture in rats whereas natural cocoa reduced infiltration of fat droplets in the hepatocytes
- Increased adipocyte sizes due to high-fat diet intake, coincided with increased fat infiltration in the hepatocytes, suggesting that enlarged adipocytes enhance the deposition of fat droplets in the hepatocytes.

- Polyphenol-rich natural cocoa decreased weight gain, improved blood glucose homeostasis, reduced adipocyte sizes, minimised liver steatosis and inhibited alterations in adipocyte and hepatocyte morphology due to high-fat diet intake.
-

5.6 Conclusion

The study showed that regular intake of high-fat diet progressively led to weight gain, impaired glucose tolerance, increased weekly blood glucose levels and altered the morphology of adipose tissue and increased fat infiltration of hepatocytes. Regular consumption of natural cocoa reduced weight gain, improved homeostasis of glucose, reduced hypertrophy of adipocytes and reduced fat infiltration in hepatocytes. It is proposed that natural cocoa intake may improve glucose and insulin levels in the blood and inhibit deleterious changes to the morphology and function of adipocytes and hepatocytes.

5.7 Limitations of the study

- The expression of TNF-α and other inflammatory markers in the adipose and liver tissues could not be estimated due to inadequate reagents for analysis.
- Serum insulin detection could not be estimated due to inadequate reagents and time constraint to carry out the analysis.
- The duration of the experiment was short.

5.8 Recommendations

- Immunohistochemistry and immunostaining techniques should be employed in further studies to determine TNF-α expression in the adipose and liver tissues.
- Further studies should include the determination of other inflammatory biomarkers such as IL-6, IL-1β and IL-1α and other proteins other than only TNF-α.

- High-fat diet should be commercially obtained.
- The duration of the experiment could be extended.

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APPENDIX I

COLLECTION OF BLOOD FOR BIOCHEMICAL ANALYSIS VIA CARDIAC PUNCTURE

- Rats were anaesthetized by ether inhalation until consciousness was lost.

- A 2 ml syringe was fixed to a 23G × 1" hypodermic needle was inserted into the left ventricle by moving about 1 cm superiorly above the xiphisternum and 1 cm laterally to the left and into the 5th intercostal space.

- The plunger of the syringe was drawn back to collect about 1.5 mL of blood into gel separation sample bottles.

APPENDIX II

FASTING BLOOD GLUCOSE (FBG) DETERMINATION

- The tail of rat was thoroughly cleaned with alcohol.
- A vein close to the tip of tail was identified and pricked with a fresh needle.
- The appropriate side of the glucose test strip (with its other end already inserted into the Safe-Accu 2 kit glucose monitor) was used to collect blood after a significant drop of blood was seen at the sight of prick.
- Blood glucose reading was taken immediately.

APPENDIX III

ORAL GLUCOSE TOLERANCE TEST (OGTT) DETERMINATION

- Weigh the rats and record.
- Fast rats for 8 hours (10pm to 6am).
- Calculate and record the volume of 20% Dextrose solution required for each rat for gavage as follows: To inject 2g of dextrose/kg body mass, the volume of the glucose gavage is 20% Dextrose (µl) = 6 x body weight (g)
- Cut the tip of the tail using clean surgical scissors. A small drop of blood (<5µl) is placed on the test strip of the blood Safe-accu glucometer for the baseline glucose level (t = 0).
- Orally gavage the rat with the appropriate volume of glucose solution.
- The blood glucose levels are measured at 30, 60, 90 and 120 minutes after glucose gavage.
- The reading were 0, 30, 60, 90 and 120 minutes.

APPENDIX IV

PROTOCOL FOR TISSUE PROCESSING (LIVER)

- Place tissue in tissue cassettes and into 50% alcohol overnight
- Remove tissue and place into 70% alcohol for 2 hours
- Transfer tissue into 90% alcohol for 1 hour
- Remove tissue and place in absolute alcohol (100%) I, II and III for 2 hours, 2 hours and 1 hour respectively
- Transfer tissue into xylene I and II for 30 minutes each
- Remove and place tissue into xylene III for 45 minutes.
- Infiltration
- Place tissue in tissue cassettes in molten wax I for 1 hour in an oven
- Remove and place into molten wax II and III for 30minutes each in an oven
- Tissues in cassettes are embedded in wax outside of oven and allowed to harden on ice.

APPENDIX V

PROTOCOL FOR TISSUE PROCESSING (ADIPOSE TISSUE)

- Incubate samples in buffered formalin overnight at 4ºC.
- Place samples in embedding cassette
- Incubate cassette in 70% ethanol at 4ºC until tissue processing is performed
- Incubate cassettes in 75% ethanol for 30 minutes.
- Incubate cassettes in 95% ethanol for 75 minutes. Repeat this step a 2nd time with fresh 95% ethanol.
- Incubate cassettes in 100% ethanol for 60 minutes. Repeat this step a 2nd and 3rd time with fresh 100% ethanol.
- Incubate cassettes in xylene for 20 minutes. Repeat this step a 2nd time with fresh xylene.
- Incubate cassette to fresh melted paraffin for 60 minutes at 60ºC.
- Transfer cassette to fresh melted paraffin and incubate overnight at 60ºC.
- Transfer cassette to fresh melted paraffin and incubate for 60 minutes at 60ºC.
- Tissues in cassettes are embedded in wax outside of oven and allowed to harden on ice.

APPENDIX VI

PREPARATION OF 10% BUFFERED FORMALDEHYDE PH 7.26

1. 10% buffered formaldehyde pH 7.26 (IL)

Formalin (37 - 40% w/v - BDH, England)……..…….100 mL

Distilled water…….. 900 mL

Sodium hydrogen orthophosphate (NaH_2PO_4)…..…….4g

Disodium hydrogen orthophosphate (Na_2HPO_4)…….6.5g

Apparatus and equipment

2. Electronic balance (Mettler CH – 8606)

3. 1000 mL flask

4. Magnetic stirrer

5. pH meter (Philips, PW9418)

6. Conical flasks and beakers

7. Plastic weighing container

8. Measuring cylinder

APPENDIX VII

PROTOCOL FOR HAEMATOXYLIN AND EOSIN STAINING

With the aid of Leica Auto Sectioner XL with the following programmed methods, the paraffin embedded heart blocks were sections at 5 micrometre prior to haematoxylin and eosin staining for histomorphometry

Staining Technique

1. De-wax sections in xylene for 1 minute.

2. Take sections to water (rehydrate) by passing them through graded series of alcohol in the order 100%, 95% and 70%

3. Stain in Haematoxylin (see below for preparation) for 15 minutes.

4. Wash in water for 2-3minutes

5. Differentiate in 1% hydrochloric acid in 70% alcohol for 1 minutes

6. Wash in water for 10 minutes

7. Stain in 1% aqueous eosin (see below for preparation) for 5 minutes

8. Rinse gently in a bowl under running tap water to wash off surplus stain.

9. Dehydrate in graded series of alcohol (75%, 95%, 100% and 100%) and keep in xylene for subsequent mounting with Dysterene Plasticised Xylene (DPX)

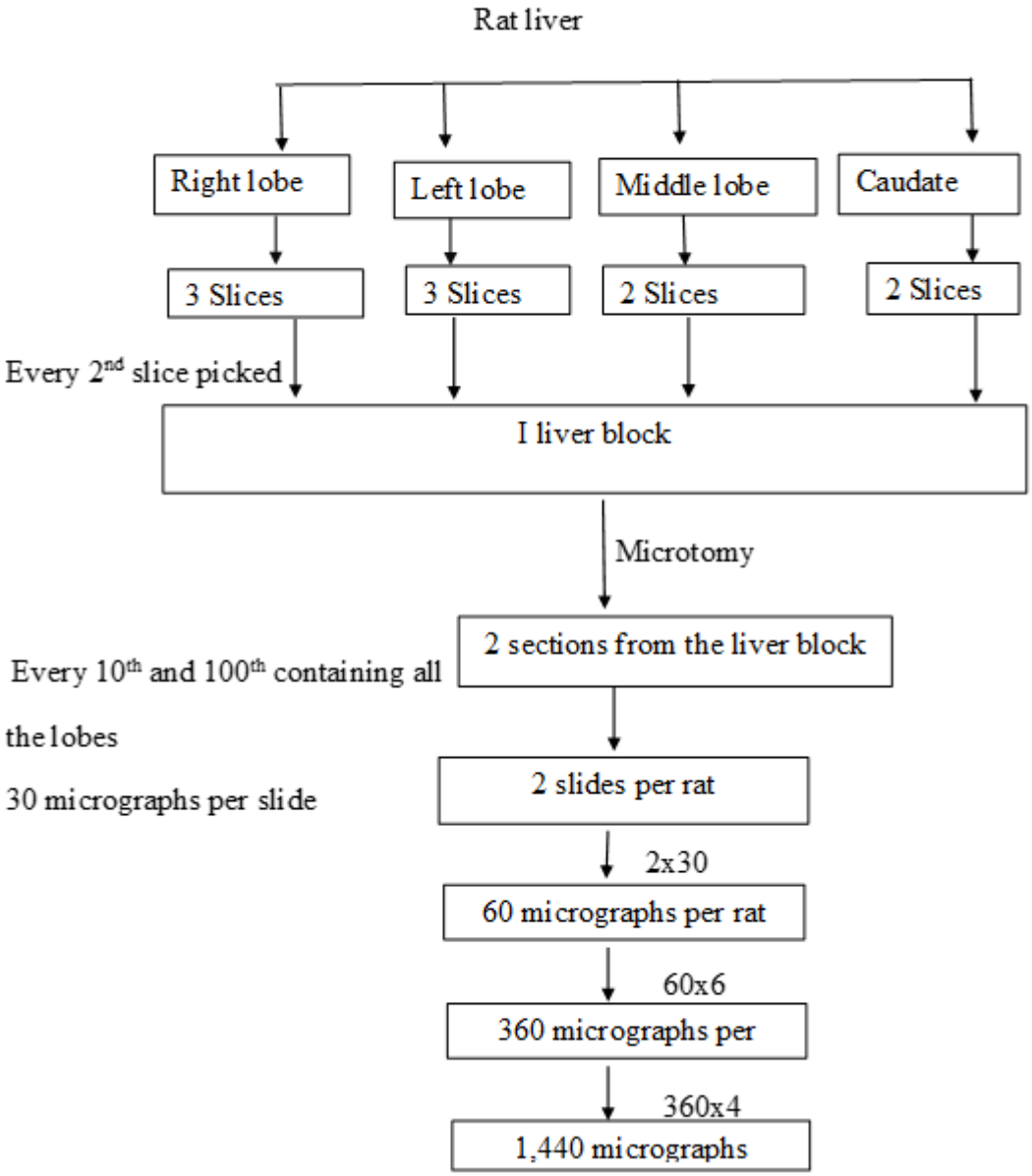
APPENDIX VIII

PROTOCOL FOR TNF-α DETERMINATION

- Reconstitute biotinylated detection antibody and protein standard and dilute the 15x wash buffer
- Perform serial dilution of protein standard and prepare samples as desired
- Add 100µl of protein standard, sample or control to each well and incubate for 2 hours at room temperature
- Aspirate protein standards, samples or controls out and wash plate 4 times
- Dilute biotinylated detection antibody as specified. Add 100µl to each well and incubate for 2 hours at room temperature
- Aspirate biotinylated detection antibody out and wash plate 4 times
- Dilute 400x streptavidin-HRP as specified. Add 100µl of 1 x streptavidin-HRP to each well and incubate at room temperature for 30 minutes
- Aspirate 1 x streptavidin-HRP out and wash plate 4 times
- Add 100µl of ready-to-use substrate to each well and incubate at room temperature for colour development.
- Add 100µl of stop solution and read plate at 450nm.

APPENDIX IX

FLOW CHART OF LIVER FOR STEREOLOGY



APPENDIX X