Potential Protective Effects of Oleanolic Acid Against High Fructose-Induced Oxidative Damage in Skeletal Muscle of Rats

S. Isaiah

orcid.org 0000-0002-7471-3877

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Supervisors: Prof L Mukwevho
Dr A O Ayeleso

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Student number: 26580748

DECLARATION

I, the undersigned, declare that this thesis, submitted to the North-West University for the degree of MSc in Biology in the Faculty of Natural and Agriculture Science, School of Environmental and Health Sciences, and the work contained herein is my original work with exemption to the citations and that this work has not been submitted at any other University in partial or entirely for the award of any degree.

Name: Simon Isaiah

Signature:.....

Date: 30/07/18

Supervisor: Prof E. Mukwevho

Signature:.....

Date:.....

DEDICATION

I dedicate this work to the Almighty God for his guidance and successful completion.

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ABSTRACT

Background: Excessive consumption of high fructose diet has deleterious effect on the metabolic system of human beings; resulting to complications in the biological system. It triggers oxidative stress and inflammation by promoting the generation of reactive oxygen species (ROS) which could lead to declining health condition.

Aim: The aim of the study was to investigate the role of oleanolic acid (OA) against high fructose-induced oxidative stress/damage in Sprague Dawley pups' rats.

Method: Twenty-four male and female suckling rats (N=24) were randomly divided into four different groups; group A (Control: distilled water), group B (OA 60 mg/kg), group C (High fructose solution (HF) (25% w/v), group D a combination of high fructose solution and oleanolic acid (HF 25% w/v + OA 60 mg/kg). The rats received the treatments once daily via orogastric gavage for 7 days at a volume of 10 mℓ/kg body mass and all suckling pups, had access to breast milk from the dams. After the treatments, all rats were euthanised after day 14 and triceps muscle tissues were collected to analysed clinical health damages. The activities of antioxidant enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) were assessed using established laboratory techniques. The gene expression of enzymes involved in oxidative stress was also done using quantitative real time polymerase chain reaction (qPCR) technique. Furthermore, biomarkers of oxidative stress such as lipid peroxidation, malondialdehyde (MDA), nitrite, total glutathione (GSH) as well as antioxidant capacity (Trolox equivalent antioxidant capacity, TEAC and ferric reducing antioxidant power, FRAP) were done using established methods. The biomarkers of inflammation were determined using Bio-Plex Pro magnetic bead-based assays. Data were expressed as mean ± standard deviation (SD) and analysed using GraphPad Prism for Windows Version 7.0 (GraphPad Software Inc., San Diego, USA) at $p<0.05$ as the accepted level of significance.

Results: The activity of catalase was increased by ~2-fold in the OA treated group but decreased by ~2-fold in the HF treated group as compared to the control group. Furthermore, the results showed a

significant increase in the inhibition of SOD activity in HF treated group as compared to control group but the inhibition was insignificant with OA and OA+HF groups. A significant increase of the enzyme GPx was observed in the OA group as compared to control group, although GPx was insignificant with OA+HF and HF groups. Regarding gene expression, there were increased expressions of CAT, GPx, SOD genes, when the rats were treated with OA as compared to the control and OA+HF groups respectively. The HF treated group was the lowest with a 5-fold decrease in the gene expression. The antioxidant capacity of the HF group increased by one-fold in TEAC assay as compared to OA, control and OA+HF groups while FRAP showed no significant difference across all treated groups. These changes in HF group were accompanied by depletion in GSH and increased lipid peroxidation, but improved when supplemented with OA. The results showed a significant 3-fold increase in GSH in OA and OA+HF treated groups with decrease in lipid peroxidation in group treated with OA+HF. Pro-inflammatory cytokines such as IL-b, IL-5, IL-6, IL-12, IFN-g, TNF- a, VEGF, and MCP-1 showed slight increased in HF group as compared to control, as well as OA and OA+HF groups. Difference was not significant. The anti-inflammatory cytokines such as IL-4, and IL-10 also showed increase in OA treated group as compared to other treated groups, although statistically non-significant.

Conclusion: Supplementation with OA attenuated fructose-induced oxidative stress/damage in the pups studied. The antioxidant potentials of OA through its ability to scavenge free radicals improved the antioxidant defence mechanism in the skeletal muscle of male and female pups. Therefore, OA has the potential and could be useful as a dietary supplement to scavenge radicals that cause metabolic oxidative damage.

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1.0 CHAPTER ONE

1.1 Introduction

Human beings face a challenge of the negative impact of the high rate of fructose sugar consumption which is a serious threat to their health (Campos and Tappy, 2016). Concerns with high consumption of fructose sugar have risen because of the realization that fructose consumption at elevated concentrations can promote health problems associated with metabolic complications. These risk complications range from cardiovascular disease, diabetes, dyslipidaemia, obesity, hyperglycaemia, and high triglyceride (Cigliano *et al.*, 2017; Stanhope, 2016). However, the behaviour of human beings and the industrialised world in modifying the human diet with sweetener to preserve and prolong the shelf life of food has played vital role in the ingestion of high fructose diets as well as the prevalence of metabolic abnormalities (Aragno and Mastrocola, 2017; Pereira *et al.*, 2017).

According to O'Neill and O'Driscoll (2015), the development of metabolic abnormalities can be traced back to lack of physical activities, ingestion of excessive fat diet and greater intake of carbohydrate sugar. Fructose is a natural sugar in many foods and cannot enter most cells unlike glucose. High fructose ingestion has been reported to cause deleterious effect to the body metabolic system (Tappy and Lê, 2010b). Its low economical cost and availability has made it an alternative usage of diet sugar than glucose in industries today (Tran *et al.*, 2009).

Skeletal muscle is one of the vital and most dynamic plastic tissues in the body system. It plays a significant role in the body and contributes to most of the body functions. But in terms of metabolic processes, it serves as storage of important macro molecules such as protein, lipids and glucose (Frontera and Ochala, 2015). High fructose intake triggers oxidative stress and thus, causes metabolic risk disorder including insulin resistance and plasma triglyceride (Taleb-Dida *et al.*, 2011). Oxidative stress can be defined as the inability of the body cell to balance or maintain homeostasis due to low antioxidant capacity which leads to high degree accumulation of ROS known as reactive oxygen species (Roberts and Sindhu, 2009). This leads to oxidative damage

thereby altering the mitochondrial bioenergetics and degradation of macro molecules in the body system (Ren *et al.*, 2010). Also, oxidative stress can result from chronic inflammation within the body tissue which is a characteristic response to continuous inflammation depending on the severity (Li *et al.*, 2014b).

Oxidative stress and inflammation are related conditions where by an increased oxidative stress results in an increase in the level of pro-inflammatory mediators which include tumour necrosis factor alpha, interleukin-6, and interleukin-1 (Sikora *et al.*, 2010). Polyphenols serve to ameliorate certain factors that endanger health and these include diabetes, dyslipidaemia and hyperglycaemia (Bahadoran *et al.*, 2013). One of such polyphenols is oleanolic acid (OA), a pentacyclic secondary metabolite found in many plant as free acid or as an aglycone, food and herbs which has shown antioxidant abilities as well as antiglycosylative properties (Xi M *et al.*, 2007). OA is a natural triterpenoid of many saponins which aid the improvement of insulin response and preserve beta cells (Castellano *et al.*, 2013). According to Wang *et al.* (2011), OA had been shown to demonstrate its protective effect against certain hepatotoxicants such as acetaminophen that cause oxidative and electrophilic stress. Studies have shown that OA contains antioxidant activity potency. It possesses the ability to lower hyperglycaemia effectively as well as serving to ameliorate high fat diet in visceral obese mice (de Melo *et al.*, 2010). The actual mechanisms through which this polyphenol brings about beneficial changes in the cells are not fully elucidated. Therefore, this study aimed at investigating the potential role of OA in regulating and scavenging excess free radicals' production. Unravelling new mechanisms of action is important and thus may provide paramount clues in the designing of effective therapy to better control or prevent the development of metabolic complications within the cell.

1.1 Statement of research problem

High fructose diet intake has been shown to alter cellular and molecular metabolic processes within the cell resulting in severe deleterious effect in the life span of an individual (Taleb-Dida *et al.*, 2011). High fructose diet elevates the production of reactive oxygen species which in turn, increases

oxidative stress and causes cell damage (El Mesallamy *et al.*, 2010). Oxidative stress causes damage to cellular functions leading to various metabolic dysfunctions. Oxidative stress itself has been linked to several abnormalities within the cell such as cancer, diabetes as well as cardiovascular diseases. Oleanolic acid as a polyphenol found naturally in various plants, may prove a vital therapeutic means to the deleterious effects of high fructose consumption. Therefore, administering oleanolic acid from natural origin can be the proper strategy in managing oxidative stress and could possibly play a role in the prevention of various diseases associated with metabolic complications.

1.2 Aim of the research study

The aim of this study was to determine the protective effects of OA on oxidative stress/damage in the skeletal muscles of Sprague Dawley pups' rats fed with high fructose diet.

1.3 Specific objectives of the research study

The specific objectives of this research study were:

1. To determine the activity of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx).
2. To determine the gene expression of the above enzymes using quantitative real-time polymerase chain reaction (qPCR)
3. To determine antioxidant capacity i.e. ferric reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC).
4. To determine oxidative stress biomarkers such as lipid peroxidation, nitrite and glutathione status.
5. To determine pro and anti-inflammatory biomarkers such as interleukins (IL-b, IL-4, IL-5, IL-6, IL-10 and IL-12), interferon gamma (IFN-g), vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1) and tumour necrosis factor-alpha (TNF-alpha).

2.0 CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 High fructose diet and oxidative stress

The consumption of dietary fructose which is known as sweetened foods is now prevalent in our society. According to Lai *et al.* (2014), "what you eat" defines your metabolic state of health. Basically, the metabolic state of an individual depends upon his/her nutritional behaviour. The upsurge of metabolic complications in humans has been linked to high consumption of carbohydrates sugar and fat diets for the past decades in the western developed countries (Joung *et al.*, 2012). Fructose is a natural sugar which serves as dietary ingredient and has the tendency to cause a rise in oxidative stress (Taleb-Dida *et al.*, 2011). This oxidative stress is defined as the inability of the body system to maintain a constant balance between the generation of reactive oxygen species (free radicals) and scavenging of reactive oxygen species that result from various metabolic pathways (Roberts and Sindhu, 2009). These ROS have deleterious consequence on basic biological molecules which include DNA, lipids, and proteins which are the building blocks that aid longevity of life span (Yoshikawa and Naito, 2002). This cause the biological system to suffer oxidative stress caused by lipid peroxidation due to the removal of electrons within the lipid membrane via the action of ROS (Castrogiovanni and Imbesi, 2012). ROS reaction also aids chronic inflammation and causes oxidative modifications of enzymes (proteins), the body's nucleic acids and deterioration of antioxidant defence mechanisms which thus affect biological functions such as regulation of redox state, its proliferation and activation which are critical for cell viability (Preta *et al.*, 2010; Li *et al.*, 2014b; Birben *et al.*, 2012). Both oxidative stress and inflammation pathways are interconnected, owning to the fact that inflammation can induce oxidative stress and the production of free radicals is a characteristic property of activated immune cells which exacerbate this accumulation (Vaziri, 2008). Thus, reactive oxygen species stimulate the activation of transcription factor such as nuclear factor kappa B (NF-$_k$ B) which triggers the production of pro-inflammatory molecule (Li *et al.*, 2014b). Skeletal muscle has shown a series of deteriorations caused by oxidative stress reaction. During ageing, it has been shown to experience cellular

dysregulation (Castrogiovanni and Imbesi, 2012). The pathogenesis of various individual illnesses can be traced to the action of both inflammation and oxidative stress due to their interactions (Ambade and Mandrekar, 2012).

2.2 High fructose, high glucose and body usage

Fructose is a monosaccharide present in many fruits and honey. Although, its chemical formula as that of glucose ($C_6H_{12}O_6$) is similar. Howbeit, the only difference in their structures is that fructose has a five atom ring with the second carbon atom having a keto group that is attached to it, while glucose is made up of a six atom ring with the first carbon having an aldehyde group (reducing) attached to it. These sugars are both simple sugars. While glucose is the main energy source for most part of the body especially the brain, fructose is mainly limited to the liver (Béland-Millar *et al.*, 2017).

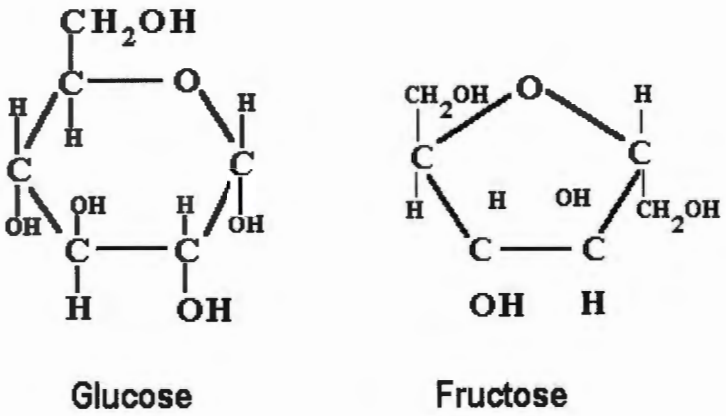

Figure 2.1: Chemical structures of glucose and fructose

In 1960, high-fructose corn syrup (HFCS) was developed as a result of invention of technologies that allow corn starch to be remolded into HFCS (White, 2014; Kim *et al.*, 2015). This HFCS contains high fructose level in it, it is inexpensive and within an acidic condition; it is stable in foods as well as in beverages. However, its consumption has increased and doubled between 1970 and 1990. HFCS contains 55% of free fructose with 42% of free glucose, while 3% portion of HFCS is other sugars, the proportion of the sugars in HFCS has significantly increased fructose consumption making a total tall of 85 to 100 g per day (Farooqui, 2013). Nowadays, HFCS is used in the manufacturing of processed foods as well as beverages in place of sucrose; these processed

products include sodas, candies, drinks, juices, cereals, dairy products, and jams, among others. The increased usage of HFCS arises from its low cost, its high concentration of sweetness as well as its ability to improve the shelf life of products (Farooqui, 2013).

It has been postulated that high level of HFCS in food can bring about increase in lipogenesis, high levels of plasma triacyclglycerols (TAGs), obesity and cardiovascular abnormalities (Rippe and Angelopoulos, 2016). When ingested in large amounts, fructose can cause hepatic insulin resistance, leptin resistance, accumulation of ectopic fat in the liver and skeletal muscle accompanied with visceral fat mass accumulation as well as increase in total fat (Rodríguez *et al.*, 2016). It is known that moderate amount of fructose does not have any negative effect, mainly because there is decrease in the response to glucose loads, as well as improvement in glucose tolerance (Laughlin, 2014).

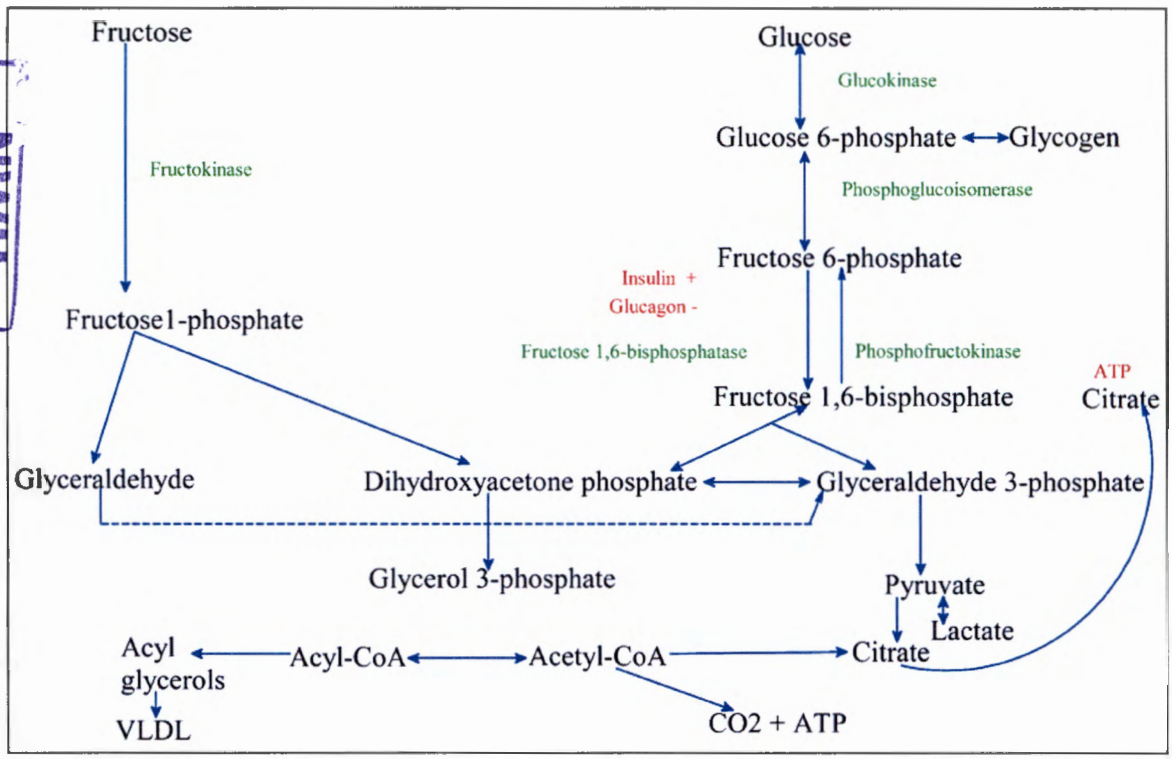

Figure 2.2: Metabolism of fructose and Glucose (Elliott *et al.*, 2002)

Fructose and glucose share many of the same intermediate structures, but have different metabolic fates in human metabolism. Both metabolisms are independent pathways; Fructose is metabolized almost completely in the liver which differs from that of glucose that passes through the liver and

goes to skeletal muscle where it is metabolized. In the initial metabolic step of fructose a critical enzyme and responses takes place; which is the enzyme fructokinase. Fructokinase catalysis the phosphorylation of fructose into fructose 1-phosphate as seen in the above (Figure 2.2). The fructose 1-phosphate is then taken up and metabolised in the liver where it is directed towards replenishment of liver glycogen and triglyceride synthesis. Thus, when the liver takes up ingested fructose, it is phosphorylated by fructokinase that initially produces fructose 1-phosphate. This is split by aldolase B to produce the trioses dihydroxyacetone phosphate(DHAP) and glyceraldehyde. An enzyme triokinase is required to phosphorylate glyceraldehyde, producing glyceraldehyde 3-phosphate. The resulting trioses are identical to those obtained in glycolysis and can enter the gluconeogenic pathway. It may produce CO_2 after being oxidized and this can further lead to the production of glucose and lactate in the biological system when it is further converted. Both lactate and glucose produced are then either allowed or enable to escape into circulation for extrahepatic metabolism, or it can be converted to fat as well as hepatic glycogen. The enormous absorption and phosphorylation of fructose within liver causes massive ATP to AMP as well as uric acid degradation (Tappy and Lê, 2010a; Malik and Hu, 2015; Sun and Empie, 2012; Bidwell, 2012). Steady fructose ingestion prompts *de novo* lipogenesis, causing high level accumulation of hepatic fatty acids in the body, and can result in ectopic liver fat when stored or deposited due to lack of usage by the body or it can be secreted as VLDL-triacylglycerols. Also, fructose impairs the extrahepatic clearance of VLDL-triacylglycerol's. Consumption of large amount of fructose in diet result in the depletion of ATP, hyper-triacylglycerolemia; thus stimulating visceral fat accumulation as well as leading to the deposition of ectopic lipid in skeletal muscle of the body (Minehira *et al.*, 2006; Lê *et al.*, 2009; Tappy and Lê, 2010a).

2.3 High fructose and metabolic syndrome

Fructose consumption at higher concentrations can also promote all the problems associated with metabolic syndrome. Metabolic syndrome is an abnormal cluster of risk conditions that correlate as a result of disrupted proper functions of the biochemical system of the body (Alam and Rahman,

2014). It is also known as syndrome X, insulin resistance syndrome, or multiple risk factor syndrome (Furukawa *et al.*, 2004). The classified risk conditions range from diabetes, hyperglycaemia, dyslipidaemia, cardiovascular diseases and obesity (De Nunzio *et al.*, 2012). Diabetes is a disease condition characterised by a severe hyperglycaemia (increase in blood glucose level in the body system) due to either defects in insulin secretion or deficient action of insulin on target tissue (Craig *et al.*, 2009). On the other hand, dyslipidaemia is an abnormality of elevated lipoproteins such as serum triglyceride, apolipoprotein B, increased small LDL cholesterol, VLDL cholesterol, while obesity refers to a condition characterised by an unusual accumulation of excess storage fats in the body system (Kassi *et al.*, 2011).

Metabolic syndrome also causes cardiovascular diseases which generally refer to conditions that lead to either partial or total blockage of the blood vessels resulting to heart failure, stroke, chest pain and heart attack (Folsom *et al.*, 2011). Globally, metabolic syndrome has recently increased with the rise in industrialised dietary foods such as apple pie, buttercream, cake, chocolate, crumble, fudge e.t.c. This has caused a challenge to public health because it is not age specific (Mozaffarian *et al.*, 2015). Fructose, which is a natural sugar in many foods, cannot enter most cells unlike glucose and its high ingestion can cause deleterious damage to the body metabolic system. Its low economical cost and availability have increased its usage other than glucose in our industries today (Tran *et al.*, 2009). Different associations and international bodies have been trying to diagnose and find the reasons behind the sudden rise in the mortality rate caused by this syndrome, but there is no diagnostic measure specific to the syndrome (Tavares *et al.*, 2015).

2.4 Polyphenols as antioxidants

Polyphenol are natural plant derivatives, some of which are obtained from dietary sources of plant origin with antioxidant potentials (Hollman *et al.*, 2011). Antioxidants have been reported to maintain and control the increase of ROS (free radicals) generated as a result of oxidative stress (Pandey and Rizvi, 2009). According to Halliwell (2008), the protective nature of polyphenol such as ascorbate (vitamin C), tocopherol (vitamin E) and carotenoids is associated with its high strength

of antioxidant activity and thus, reduces the danger of developing age related illness including cardiovasular disease, dementia and cancer. It aids cell survival and longevity by playing a key role in the scavenging of free radicals (Scalbert *et al.*, 2005). An example of such polyphenols in food and plants is oleanolic acid. Oleanolic acid, a natural triterpenoid has been shown to ameliorate oxidative injury induced by tert-butyl hydroperoxide (Wang *et al.*, 2010). It has anticancer properties by interfering in the development of cancer as well as directly modulating enzymes that are linked to insulin biosynthesis, signalling and secretion (Dzubak *et al.*, 2006). Research showed that it has great pharmacological properties such as antidiabetic, anti-atherosclerotic, anti-inflammatory as well as antioxidant potentials (Castellano *et al.*, 2013; Wang *et al.*, 2011). However, its mechanisms of action through which it confers these properties are not fully understood.

2.5 Free radicals and deleterious effect

Free radicals are molecules that have one or more unattached electrons. The presence of these electrons makes the radicals become unstable, thereby inducing more reactivity in them. Production of free radicals by biological substances was first reported in 1954 (Powers *et al.*, 2011). Endurance exercise induces passive oxidant agitation in skeletal muscles generates large quantities of free radicals, resulting in the oxidative capacity of skeletal muscles thus, outstrip its endogenous antioxidant enzymes and insulin sensitivity (Ferreira *et al.*, 2010). This supports the findings in the hormesis hypothesis in the sense that adaptations through exercise occur by the activation of signaling pathways leading to increased production of antioxidants (Gerry *et al.*, 2008). However, these free radicals, which are also by-products of biochemical metabolic processes in the body are toxic and cause cytotoxic damage to body cell and tissues, are made non-toxic by the antioxidant. Free radicals which induce deleterious effect in biological system comprise of superoxide radical, hydrogen peroxide, nitric oxide, hydroxyl radical, hydroperoxyl radical, hypochlorite ion, singlet oxygen but those of high effect are collectively known as reactive oxygen species (Fang *et al.*, 2002; Yoshikawa and Naito, 2002).

Generally, the outer orbit of these molecules have an unpaired free shared electron which makes them very reactive and unstable (Gilbert, 2000). In aerobic life the production of free radicals such as reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive chlorine species requires the availability of oxygen and are generated persistently via normal cellular metabolism in the biological system while some of these radicals are also induced by activated cytokines (Roberts and Sindhu, 2009; Fang *et al.*, 2002; Castrogiovanni and Imbesi, 2012). At increasingly accumulation reactive oxygen species are deleterious with a devastating consequence on cell membranes, including phospholipids, other major organelles which include lysosomes, mitochondria, DNA as well as nucleotides, it aid in enzymes degradation as well as impede cell proliferation and migration (Pushparani, 2015).

Superoxides are oxygen derivatives and are produced via aerobic metabolism in the mitochondrial electron transport system. The production of NADH, NADPH and $FADH_2$ as a result of dietary intake thus, increases the release of free radical (Fang *et al.*, 2002). Furthermore, ROS are extremely reactive cytotoxic agent damaging cellular structure and cause dysregulation of the body metabolism by inducing deterioration in the form of lipid peroxidation, nuclei acid damage, oxidative modification of protein molecules (Banerjee *et al.*, 2003; Preta *et al.*, 2010). One of the amino acids in the muscle that is involve in cell division is glutamine. It is found in the blood and degraded to glutamate. The glutamate serves as a substrate for nucleotide biosynthesis, glucose synthesis or as fuel (ATP production) in the body. However, elevated levels of free radicals in the biological system, cause a drastic decrease in glutamine, thus influencing immune function and altering the availability of glutamine to leucocyte and result in low muscular performance (Walsh *et al.*, 2000; Kinnunen *et al.*, 2005). ROS play significant contribution in most of the human pathological disorder in patient with high risk of complication caused as a result of oxidative stress within body system (Atalay *et al.*, 2006). In the cell, the main free radicals formed are superoxide (O_2^{-}.) that are produced via partial reduction of oxygen in electron transport system, and nitric oxide

(NO) which is produced through enzymatic reactions. Some of the vital ROS are recapitulated below.

Table 2.1: Other free radicals and its effects in biological system.

Free radicals	Characteristics	References
Superoxide	Commonly produced as biochemical reactions intermediate that is impermeable to cell membranes and its negatively charged with hydroxyperoxyl radical being an exception. It has long half-life and can be produced by many cells when fighting against pathogens. Dismutation of hydrogen peroxide in the cells by superoxide dismutase helps to prevent damages within the biological system.	(Sies, 2014; Liochev, 2013)
Hydroxyl radicals	They are very reactive due to their strong oxidizing potential. They would normally cause damage to surrounding molecules. They are the most damaging ROS and can be said to be non-existing if not for the presence of some products of their reactions. They are not permeable.	(McMurray *et al.*, 2016; Losada-Barreiro and Bravo-Díaz, 2017)
Singlet oxygen	Another essential ROS with limited half-life yet; permeable to cell membranes. It is oxygen in its excited state but without unpaired electrons so it cannot be termed a radical. In water, the dismutation of superoxide anion brings about the production of highly oxidizing singlet oxygen.	(Ribou, 2016; Apak *et al.*, 2016)
Hyperchlorite	It is formed because of the activity of myeloperoxidase	(Winterbourn and

	in the biological system using hydrogen peroxide. It is frequently created via the action of neutrophils and hazardous to thiols, ascorbate, lipids, and NADPH. In acidic form, it is permeable to cell membranes.	Kettle, 2013; Davies, 2010)
Peroxynitrite	This is produced from the reaction between nitric oxide and superoxide. It is a fast reaction even more rapid in reaction than nitric oxide with heme proteins. It is a strong oxidizing agent that can damage DNA, reduce thiol groups and subsequently cause protein damage.	(Subelzu *et al.*, 2015; Denis, 2015)

2.6 Antioxidant enzymes and cellular longevity

The longevity of a life span in relation to age-related diseases is dependent upon certain factors that are responsible in the prolonging of body cells (Sadowska-Bartosz and Bartosz, 2014). These include free radical reactions, ROS which is the main factor, while others may involve reactive aldehyde errors in protein synthesis that arise from biochemical processes (Rattan, 2013). Aging is caused as a result of the accumulation of molecular damages to body cells and tissues (Zimniak, 2011). Because of their potential harmful effects of ROS, excessive ROS must be promptly neutralized and eliminated from the cells by a variety of antioxidant defence mechanisms. Increase in calorie increases the activity of mitochondrial aerobic metabolism thus producing more free radicals while calorie reduction retard aging as well as reduces free radicals production (Fang *et al.*, 2002).

There is a need to prevent damages resulting from aging. This can either be achieved by synthesizing antioxidants *in vivo* in the body cell or obtaining it via dietary source from the environment. Antioxidants are known to maintain stability and control the increase of free radicals caused via the action of oxidative stress in the biological system (Roberts and Sindhu, 2009).

Antioxidants are molecules that contain free electrons that can be donated to stabilize ROS (Flora, 2009; Lobo *et al.*, 2010). Antioxidants include both hydrophilic and lipophilic molecules for metabolizing ROS. Therefore, antioxidants prolong cellular longevity by slowing down aging in the life span of an individual. These can be derived from external sources (exogenous antioxidant) or generated from within the biological system of the body (endogenous antioxidant). These defence mechanisms against ROS can be grouped into three antioxidant pathways which include intracellular, extracellular and membrane antioxidants. Intracellular ROS scavenging enzymes (FRSEs), which is the primary system include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). SOD decreases the excessive level of ROS to non-toxic reactive H_2O_2, which is then further detoxified into water via the action of CAT with GPx enzyme. GPx is one important enzyme in terms of lipid peroxidation as well as the scavenging of OH^- (Pushparani, 2015). Detoxification of ROS is controlled by extracellular or membranous antioxidant and compounds such as Vitamins A, C and E, glutathione, NADPH which serve as the secondary system (Bainbridge, 2013).

2.6.1 Endogenous antioxidants

These are cellular antioxidants that are present in the biological system and play a vital role in maintaining redox balance, thus causing stability in homeostasis (Gul *et al.*, 2000). These endogenous antioxidants enzymes mainly include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR). Scavenging of free radicals involves the ability of cells to maintain constant level of these enzymes, thus preventing aging (Roberts and Sindhu, 2009). Superoxide dismutase enzyme catalyses the partitioning of superoxide (O_2^-) which are biologically toxic and deleterious when produced as by-product of mitochondrial metabolic processes into hydrogen peroxide (H_2O_2) and/or harmless molecular oxygen (O_2). Catalase is an enzyme present in the peroxisomes of eukaryotic cells that causes the degradation of hydrogen peroxide (H_2O_2), a cell damaging agent produced during aerobic metabolism thereby completing the detoxification reaction initiated by SOD. GPx and GR enzymes are those that degrade hydrogen

peroxide and also reduce organic peroxides, thus helping in creating route for eliminating toxic oxidants in the biological system (Herken *et al.*, 2001; Erkiliç *et al.*, 2003; Szymonik- Lesiuk *et al.*, 2003). But in an individual that is not well nourished where the biological function of the body is unable to metabolised these toxic substances, then the need for dietary source to supplement in scavaging of free radicals is of paramount thus avoiding the risk of damage.

2.6.2 Exogenous antioxidants

Exogenous antioxidants are diet derived supplements obtained from plants source and they are of natural origin (Pandey and Rizvi, 2009). These help in scavenging oxidative stress biomarkers in the biological system when enzymatic antioxidant are insufficient to control the net amount of free radical production that exceeds its capacity within the body system, thus maintaining stability (Williamson and Manach, 2005; Keen *et al.*, 2005). Dietary exogenous antioxidants help to control the level of ROS in order to minimise oxidative damage (Halliwell and Gutteridge, 2007).

These active metabolites that reduces the concentration of ROS which is toxic to the body are gotten from a variety of food and beverages such as coffee, tea, vegetables, fruits, cocoa shells, olives, garlic, ginger, red onion skin, grapes, apple cuticle, nutmeg, mustard leaf seed, peanut seed coat and are used as complementary medicine supplements (Hollman *et al.*, 2011; Aruoma, 2003). Some of these main metabolites include vitamins C, vitamins E and vitamins A, β-carotene, polyphenols, carotenoids, catechin, Epigallocatechin gallate (EGCG), flavonoids, and selenium (Scalbert *et al.*, 2005). They all contribute to protecting the cells against free radical damage. Vitamin E, also known as tocopherol, is widely distributed in nature and it is the primary antioxidant in cell membranes (Abadi *et al.*, 2013). Vitamin C (ascorbic acid) is hydrophilic, thus making it effective in aqueous environment. Ascorbic acid directly scavenges free radicals as well as playing a part in vitamin E recycling (Rodríguez-Roque *et al.*, 2015).

Carotenoids are lipid soluble antioxidants, their structural arrangement allows for the scavenging of free radicals (Masisi *et al.*, 2016). According to Maestri *et al.* (2006), these metabolites have shown in past studies that they contain antioxidant properties which are used as therapy in the control of a

number of metabolic diseases. For optimal level of defence and protection from oxidative damage, a biological system will need supplementary exogenous antioxidant which is seen as a potential prophylactic agent that can aid in terms of health and disease management (Pryor 2000). This has drawn much attention from both the food industry and local consumers with specific attentiveness to the family of the polyphenols because they are present almost everywhere, thus potentially elucidating the high intake of fruits and vegetables (Hollman *et al.*, 2011).

2.6.3 Antioxidants in oxidative damage prevention

There are two categories of antioxidants, namely enzymatic and non-enzymatic antioxidants. They are discussed subsequently. Enzymatic antioxidants: The main antioxidant enzymes are superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase.

2.6.3.1 Superoxide dismutase (SOD):-

This enzyme which was discovered in 1969 protects against superoxide radicals. The function of SOD is the dismutation of superoxide to form hydrogen peroxide and oxygen.

$$2O_2^{\bullet-} + 2H+ \xrightarrow{SOD} O_2 + H_2O_2$$

Three isoforms are reported with each having a core metal at its catalytic site which activates the breakdown of superoxide anions (Davis *et al.*, 2017; Cheng *et al.*, 2014). One is present in the extracellular space, while the other two are within the cell. The first isoform which is located in the cytosol and mitochondria has copper-zinc as its cofactor, the second sited in mitochondrial matrix has manganese as its cofactor while the third located in the extracellular space makes use of copper-zinc as its cofactor (Huseynova *et al.*, 2014). Furthermore, superoxide radicals are not known to be extremely reactive, they have been shown to effectively extract electrons from membranes causing electron imbalances in biological membranes through the production of free radicals (Hung *et al.*, 2014). This necessitates the need to keep super oxides in check. Mutations in the first isoform of SOD have been reported to cause apoptosis of spinal neurons leading to amyotrophic lateral sclerosis (Tan *et al.*, 2014). In skeletal muscle fibers, about 15 to 35% activity of total SOD is actualize inside the mitochondria with about 65-85% remaining within cytosol (Cheng *et al.*, 2016).

In the oxidative fibers; these activities to a great extent are significant (e.g. type I fibers) when compared to those muscles whose volumes of mitochondria are low (e.g., type IIx fibers).

2.6.3.2 Glutathione peroxidase (GPx):-

Glutathione peroxidase was reported that about five different mammalian GPx exist in the biological system (Malandrakis et al., 2014; Wang et al., 2017). They function basically in the catalysis of hydrogen peroxide to water, or alcohol depending on whether the hydrogen peroxide is organic or not using reduced glutathione (GSH) (Wirth, 2015).

$$H_2O_2 + 2GSH \xrightarrow{GPx} 2H_2O + GSSG$$

The reduced GSH are concomitantly oxidized to glutathione disulfide (GSSG) after donating electrons. Each form of GPx is substrate specific though they carry out similar reactions. The reality is that GPx can decrease various hydroperoxides, making them crucial intracellular antioxidants in preserving the system against the action of ROS-mediated cell deterioration (Huang et al., 2016). The reduction of GSSG back to GSH after it has been reduced is carried out by the enzyme glutathione reductase using NADPH as the energy source (Huang et al., 2016).

2.6.3.3 Catalase (CAT):

Catalase breaks down hydrogen peroxide by catalyzing it to water molecule and oxygen.

$$H_2O_2 + H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

CAT is widely distributed having iron as its cofactor attached to its active site (Cheng *et al.*, 2014). CAT has a lower affinity for hydrogen peroxide compared to GPx (i.e., GPx Km = 1μM vs. CAT Km = 1mM), but it activity rate in an extremely oxidative muscle fibers increases and low in fibers muscle with decreased oxidative capacity compared to both SOD and GPx (Huang *et al.*, 2016; Bunpo and Anthony, 2015).

Along the primary antioxidant enzymes, other additional enzymes that participate in redox balance are also present. These include the thioredoxin (TRX), peroxiredoxin (PRX) and glutaredoxin (GRx) (Hanschmann *et al.*, 2013; Mahmood *et al.*, 2013; Rhee and Woo, 2011).

2.6.3.4 Non-enzymatic antioxidants:

Reportedly, the most important muscle fiber non-enzymatic antioxidant is GSH, and it is the most abundant non-protein thiol in cells (Schmitt *et al.*, 2015). GSH which is produced in the liver varies in concentration across different organs based on their functions (Petry *et al.*, 2015). That is why tissues with oxidants contain more amounts of GSH. The GSH in skeletal muscles is based on the fiber types as shown in type I fibers in rats which contain 4-5 fold greater amount of GSH compared to type IIb (Steinbacher and Eckl, 2015).

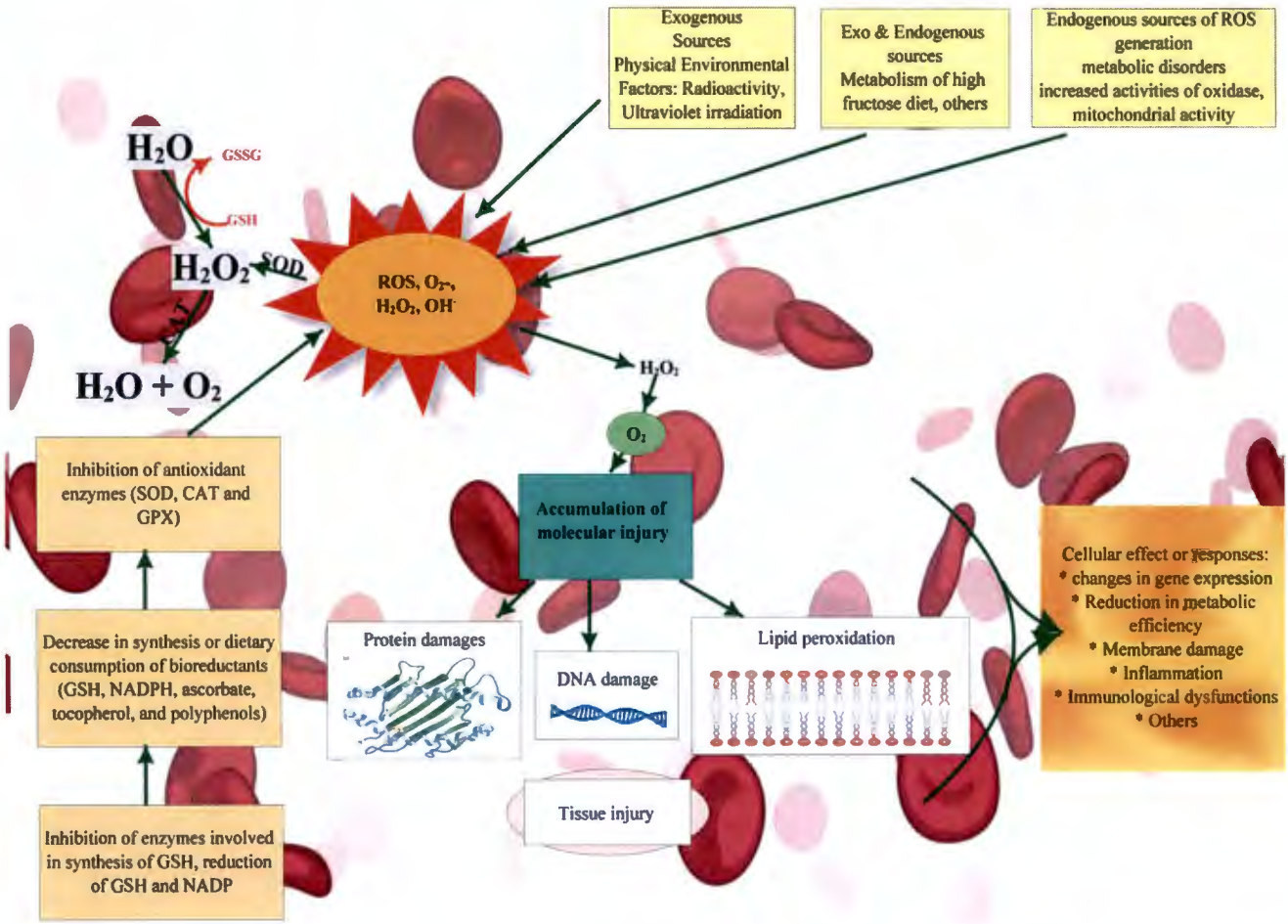

Figure 2.3: Source of free radical production and damages to biological system (Roberts and Sindhu, 2009; Yoshikawa and Naito, 2002; Gospodaryov and Lushchak, 2012)

Many studies showed that skeletal fibers have the ability to remodel themselves in climax intensity of tolerance during exercise when they increase glutathione (GSH) magnitude in the cell (Gomez-

Cabrera *et al.*, 2016; Steinbacher and Eckl, 2015).Tthis process is due to increased activity of ɣ-glutamylcysteine synthetase known as the rate limiting enzyme; which is GSH biosynthesis (Couto *et al.*, 2016).

Another important non-enzymatic antioxidant is α-lipoic acid, a natural compound present in a variety of foods (Rochette *et al.*, 2013). It serves as cofactor for α-dehydrogenase complexes and participates in some cellular reactions as well. It has been concluded by many studies that α-lipoic acid can recycle vitamin C (Pingitore *et al.*, 2015). Light exercise may increase its level in skeletal muscle fibers, but prolonged and constant exercise does not (Pingitore *et al.*, 2015). Other known non-enzymatic antioxidants are uric acid, bilirubin, biliverdin, and coenzyme Q10 (Silvestri *et al.*, 2015).

2.7 General overview of muscle

All forms of movement are important for systems maintenance, protection, stability, translocation and reproduction in vertebrates. All forms of movement will never be possible without the actions of the muscular system. The muscle consists of a large percentage of the entire body mass. It is heterogeneous and contains both light chain and heavy type 1 and 2A, 2X such as myosin, tropomyosin, troponins and actins complexes (Dubowitz *et al.*, 2013). It also comprises of most of the proteins of metabolism taking part in excitation-transcription coupling. Muscle can adjust its size and function in response to internal and external feedbacks. About 630 muscles found in humnas make up 40% of the total body weight and contain about 50-75% of all body protein as well as 5% of other substances which include fat, carbohydrates, minerals and inorganic salts (Pearson, 2012; Frontera and Ochala, 2015).

2.7.1 Skeletal muscle

The origin for the build-up and regulation of force for locomotion is provided by the skeletal muscle (Goldmann, 2014). They support the body in many ways such as maintenance of posture; they produce movement that influences activity. They are a storage for important substrates such as amino acids and glucose carrying the whole-body weight in all the different postures like standing and sitting, as well as being the focal point when an organism is in motion. The skeletal muscle can be said to be the main muscle that the body relies on. It is very relevant when it comes to posture and every other activity that involves movement of the organism. It majorly connects with the bone to carry out its functions (Schickert, 2014). Skeletal muscle comprises of some well-defined units that are attached to the bones or other muscles through the tendon and ligaments respectively which help in supporting special locomotion processes. Apart from its function in locomotion, skeletal muscles have been found to be a major factor to be considered when it comes to the control of glycaemia and metabolic homeostasis (Volpi *et al.*, 2004). It is the predominant site for glucose disposal in insulin mediated conditions. Along with the liver, skeletal muscle stores glycogen as it has four fold capacity more than the liver making it the highest glycogen reservoir in the body

(Sullivan, 2014). There are several types of skeletal muscles which have distinct biochemical properties (Ciciliot *et al.*, 2013) .

2.7.2 Skeletal muscle and its significant role in the body

Skeletal muscle is different from other muscles in the sense that it is the single one of its kind that can sporadically cause an increase in its energy consumption when there is need for sudden and volatile contractions. This energy increase level can rise to 300-fold from the resting state to the fully active state which takes just few milliseconds to occur as it is extremely fast (Mendias *et al.*, 2017). This ability to speedily increase its rate of energy production and flow of blood which usually takes place in response to locomotion solely distinguishes the skeletal muscle from others. Movement or locomotion is caused by the sliding filament theory of the actin-myosin cross-bridge during the skeletal muscle contraction (Morrow, 2011). The energy in the cross-bridge is provided by the hydrolysis of adenosine triphosphate (ATP) with myosin ATPase being the enzyme involved (King *et al.*, 2004). In metabolism, changes occur due to different activities going on in the body. One of this changes is caused or induced by exercise and this type of change due to exercise is majorly carried out by the skeletal muscle (Triantou, 2015). During exercise, oxygen consumption increases to about 30-fold as well as blood flow. Tricarboxylic acid (TCA) activity also increases to about 70 to 100 fold (Dash *et al.*, 2007). The energy used by the skeletal muscle which is in the form of ATP is gotten majorly from oxidative phosphorylation. This is made very possible and easy due to the high presence of mitochondria in the skeletal muscle (Gibala, 2016). In the production of energy from carbohydrate and lipid metabolism, skeletal muscle is the main site though its actions can be regulated by the actions of the contractile bioenergetics involved.

2.7.3 Effect of oxidative stress on skeletal muscle

Skeletal muscles are a vital and most dynamic plastic tissues and constitutes the largest insulin-sensitive tissue as well as being paramount in most of the body functions. The vulnerability of the body high ingestion of fructose causes oxidative stress in the skeletal muscles. Majority of

researchers have shown that oxidative stress can be deleterious to cells and is a major cause of most chronic diseases known. Why is oxidative stress so detrimental and what causes it?

Oxidative stress is the damage caused to cells, tissues or organs by reactive oxygen species (ROS). ROS are produced from the free radicals which are present in cells. Although free radicals are regarded as deleterious, they have been found to be of help in the control of signaling pathway, control of gene expression and in the modulation of muscle force generation (Viña *et al.*, 2016). Alternatively, oxidative stress has been redefined as the macromolecular oxidative damage resulting from the disruption of redox signaling and control (Navarro-Yepes *et al.*, 2014). Simultaneously, other metabolic pathways are compromised due to the generation of ROS in the skeletal muscles (Beckendorf and Linke, 2015; Sakellariou *et al.*, 2014).

Myofibrils are affected by oxidative damage during prolonged exercise in skeletal muscles due to the effect of muscle contractions which produces free radicals (Wong *et al.*, 2017). In early event of apoptotic pathway triggered by ROS in oxidative stress program, the death of mature skeletal muscle cell also causes death of progenitor (Barbieri and Sestili, 2011). Owing to the necessity of maintaining a state of normality in cells, antioxidant defense mechanisms are available in cells including skeletal muscle fibers in order to suppress damages due to oxidative stress as a result of the production of ROS (Cheng *et al.*, 2016). Antioxidants maintain a balanced physiological ROS levels where they can function in signal transmissions (Bloomer *et al.*, 2006). These antioxidants are well organized and function effectively to prevent the generation of free radicals (Anjum, 2015). These antioxidant enzymes affect the maturation of skeletal muscle cell and tissues regeneration (Hidalgo *et al.*, 2014).

2.7.4 Skeletal muscle and effect of antioxidant

According to Silvestre *et al.* (2013), the significant function of antioxidant defense enzymes in the development of new blood vessels (angiogenesis) and regeneration of muscle are interconnected in that, the induction of angiogenic response occurs as a result of the disruption of oxygen supply in a study of hind limb ischemia model carried out. Both angiogenesis and fibrosis are not considered as

part of the restoration of skeletal muscle constituent, but the events are actually correlated. When vast muscle progenitor cells are restricted closer to blood vessels, the muscle becomes more vascularised. Therefore, during regeneration or repair of injured tissue muscles, it is unsurprising that it involves concurrent tissue revascularisation to restore blood supply (Bentzinger *et al.*, 2013; Yin *et al.*, 2013). Previous report, indicated that the inhibition of capillary development due to high degree of $O_2^{\cdot -}$ generation was shown because of SOD^3 deficiency in an experimental setting (Kim *et al.*, 2007). These endogenous antioxidant enzymes are known to obstruct the development of fibrosis within the body skeletal muscle. Galasso *et al.* (2006) reported that the deficiency of GPx-1 protein hinders the rehabilitation of blood flow. It was reported that the consequence of antioxidant enzymes on skeletal muscle regeneration are imputed to the accumulated viability of myogenic precursors under oxidative stress. This protective prospective was indicated in vivo for GPx and CAT may inhibit myogenic proliferation (El Haddad *et al.*, 2012; Lee *et al.*, 2006; Kozakowska *et al.*, 2015). However, besides the enzymes found in mammalian cells which represent the basic primary endogenous antioxidant, there are other enzymes that constitute the second phase of antioxidant defence in skeletal muscle cells. These include γ-glutamyl cysteine synthetase (GCS) and heme oxygenase-1 (HO-1). They do not play a sole part in the scavenging of ROS, but also play a role in synthesis of non-enzymatic antioxidants that are present in skeletal muscle such as GSH by GCS or biliverdin and bilirubin by HO-1 (Powers *et al.*, 2011; Kozakowska *et al.*, 2015).

2.8 Inflammation and cytokines response to tissue damage

Inflammation is the body's immune responses to stimulus which can either be pathogenic effects of chemicals produced in the body (toxin) or radiation. Cytokines are secreted proteins which are small in size that are released by cells with a specific effect between cells to aid in interactions and communications (Zhang and An, 2007). Cytokines are known to stabilize inflammatory condition and preserve lymphocytes homeostasis (Sanjabi *et al.*, 2009). Tissue damage occurs as a result of oxidative stress. It increases inflammatory mediators in the body system because both pathways go hand in hand and thus are inseparable and are interconnected. They are pivotal in the pathogenesis

of several human diseases and help majorly in most diagnostic procedure (Li *et al.*, 2014a). Because diverse number of inflammatory cytokines are prompted by the action of oxidative stress, the reality is that these cytokines themselves trigger the release of other cytokines which also lead to aggravated oxidative stress which make them crucial in chronic inflammation (David *et al.*, 2007; Chokkalingam *et al.*, 2013). Increased level of cytokines circulation in the body and pro-inflammatory biomarker can also be associated with age-related changes (Michaud *et al.*, 2013). However, inflammatory mediators and cytokines are key in the cutaneous process of wound healing, tissue remodelling as well as in either tissue damage or surgical injury which can also aid the survival of individual in an acute and chronic state/condition (Hsing and Wang, 2015). Cytokine also plays a major role acting as regulator of host response to inflammation, although, some tend to worsen (pro-inflammatory mediators) the state of the disease condition as well as stimulating systemic inflammation while others minimize (anti-inflammatory mediators) the effect cause of the inflammation and aid in the healing process (Dinarello, 2000a).

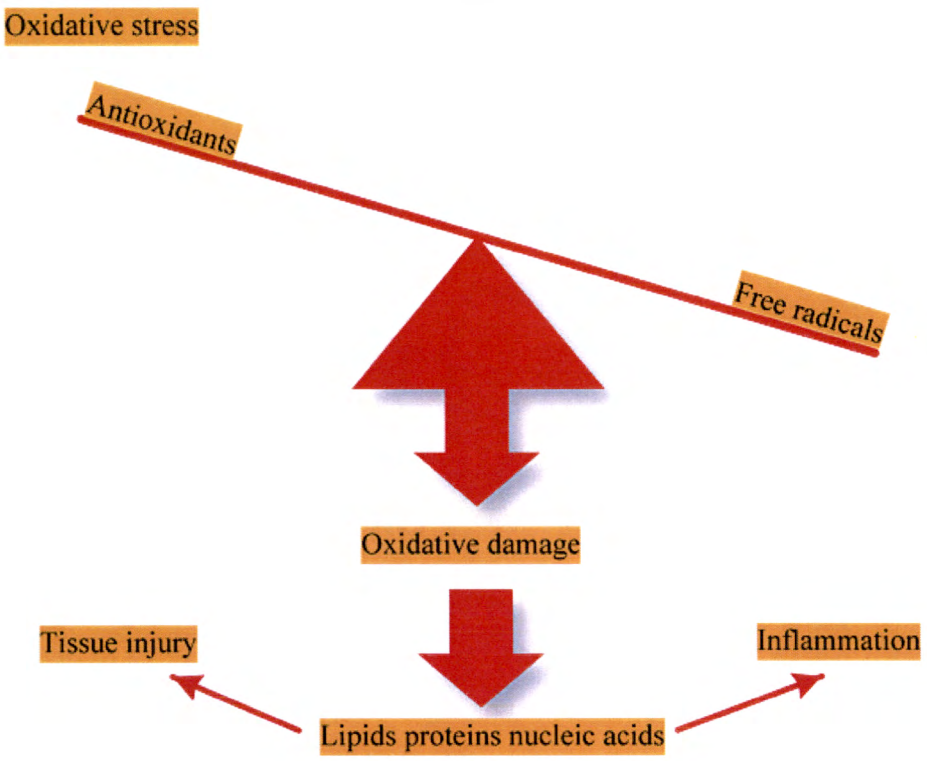

Figure 2.4: The relationship between oxidative stress and inflammation (Kelly, 2003)

2.8.1 Pro-inflammatory cytokines

These are inflammatory cytokines (signalling molecules) that are secreted from immune cells such as T-helper cells, macrophages and some other kind of cells that stimulate inflammation. According to Dinarello (2000b), proinflammatory cytokines effect produces fever, tissue destruction and in some exceptional cases leads to shock and even result in death when they are administered to humans. However, they are mainly generated following the activation of macrophages and thus play a role in the complementary process of inflammation reaction. These include IL-1β, IL-6, TNF-α, MCP-1, IL-12, IL-17, IL-8, IL-23. However, preventing the expression as well as blocking the activity of the proinflammatory cytokines IL-1, TNF, IL-6, IL-12, IL-17, IL-8, or IL-23 which mitigate inflammation and suppresses activated T cells, alters specific pathways that restrain the expression of T-cell activation (Dinarello, 2010). Interleukin-1 beta (IL-1β), which is also referred to as catabolin is secreted primarily by monocytes, macrophages and by endothelial cells, as well fibroblast cells which are non-immune during injury to cells, inflammation, even invasion. Pro-inflammatory cytokine like the IL-1β generates hyperalgesia during its expression, consequently after the injection of intraperitoneal or intracerebroventricular injection (Özaktay *et al.*, 2006). It is also enhanced when there is a crush injury to peripheral nerve and thus raise the production of principal mediator of inflammation diseases known as prostaglandin E_2 (PGE_2) in rheumatoid arthritis and osteoarthritis (Park *et al.*, 2006). One key cytokine that induces injury is IL-6. It is basically secreted from T cells and macrophages to cause a stimulating immune reaction to trauma, particularly burns or causes other tissue damages that lead to eruption such as inflammation and when there is an Interleukin-6 (IL-6) imbalance. During production by the body it promotes immune dysfunction. Because it is a pleiotropic cytokine, it is secreted in reaction to stimuli inflammation although, IL-6 can mediate the inflammation of both organ and tissues damage (Umare *et al.*, 2014; Chaudhry *et al.*, 2013). IL-6 is a fundamental regulator of cellular processes, neuronal reaction to nerve injury, bone metabolism, and neuronal degeneration. It is also involved in microglial and astrocytic activation (Heijmans-Antonissen *et al.*, 2006).

TNF-α is another pro-inflammatory, a 17 kDa protein derived predominantly from activated immune cells (macrophages) as well as from nonimmune cells (fibroblasts) in response to invasive, infectious, or inflammatory stimuli. This cytokine plays a well-established key role in few pain models. Is is also known as 'cachectin' which acts on several different signalling pathways via two cells receptors (TNFR, TNFR2), thus regulating apoptotic path ways as well as NF-kB activation of inflammation (Zhang and An, 2007; Parameswaran and Patial, 2010). The expression of several molecules in the biological system needed for acute inflammation including proinflammatory cytokines such as IL-1, IL-6, IL-8 and chemokines MIP-1, MCP1, RANTES are controlled by Activated NF-kB (Rock and Kono, 2008). Both IL-1 and TNF-α instigate a cascade of mediators which are directly responsible for the various events associated with inflammation and orchestrate the inflammatory response (Cavaillon, 2001).

IL-8 is a chemokine that serves as a chemical signal that attracts neutrophils to the site of inflammation. It serves as a mediator in terms of inflammatory response; indicating its role as a vital member of anti-inflammatory cytokines. It causes the induction of chemotaxis in target cells as its main primary purpose, and it is produced by macrophages, epithelial cells, endothelial cells as well as other cell types. IL-12 triggers the production of IFN-γ and TNF-α from T- and natural killer (NK) cells by stimulating these cells. It decreases IL-4 mediated prevention of IFN-γ. IL-17 is generated by helper T-cells and is persuaded by the IL-23, and thus leads to the destructive damage of tissue in chronic inflammation. It also plays a vital role in stimulating the generation of other cytokine such as IL-6, IL-1β, TNF-α, and monocyte chemotactic protein-1 (MCP-1), which serve as potent mediator in regulating inflammation, thereby causing an elevated level of chemokine secretion in different tissues to recruit both monocyte and neutrophils that migrate to inflammation site. Another member of anti-inflammatory cytokine is IL-17 which is also entailed in excessive tissue damage (Hu *et al.*, 2011; Shahrara *et al.*, 2010; Witowski *et al.*, 2000; Costa *et al.*, 2010; Schoenborn and Wilson, 2007; Chaudhry *et al.*, 2013; Reynolds *et al.*, 2010).

2.8.2 Anti-inflammatory cytokines

These are a chain of molecules that are immunoregulatory that act as vital antagonist against tissue damage. They counteract various aspects of inflammation and control pro-inflammatory cytokine response (Wynn and Vannella, 2016). However, their physiological role in inflammation as well as pathologic role in systemic inflammatory condition are being increasely (Zhang and An, 2007; Wick *et al.*, 2010). They intercept the potentially harmful effects of persistent or increasing inflammatory reactions. The main cytokines that aid healing include interleukin (IL)-1 receptor antagonist, IL-4, IL-10, IL-11, IL-13 interferon alpha (IFN-α) and transforming growth factor-β (TGF-β). Because of their ability to restrain the secreting of harmful cytokines, they induce the fabrication antagonist such as IL-1 receptor (IL-1ra). They also allow soluble TNF receptor (sTNFR) as well as to limit some of the proinflammatory activities of IL-1 and TNF (Chaudhry *et al.*, 2013; Cavaillon, 2001). But the key anti-inflammatory cytokine is IL-10 generated by $CD4^+$ Th2 cells. Monocytes and B-cells strongly impede the expression of both IL-2 and IFN-γ as well as suppresses the fabrication of TNF-α, IL-1, IL-6, IL-8, IL-12, in monocytes, macrophages, neutrophils and NK cells (Opal and DePalo, 2000). According to King *et al.* (2014), IL-10 has been demonstrated to play a paramount role in a variety of other models of tissue repair beyond cutaneous wound healing, fetal regenerative wound repair as well as in re-establishing tissue integrity in a tendon injury model. (IL)-4 can interfere in the fabrication of tumour necrosis factor (TNF)-α and IL-1β by lipopolysaccharide (LPS)-activated human monocytes. It also induces the fabrication of the IL-1 receptor antagonist (IL-1ra) (Woodward *et al.*, 2010).

2.9 Oleanolic acid

oleanolic acid is a natural pentacyclic triterpenoid of saponins component found in many plants as free acid, food and herbs (Fai and Tao, 2009). Terpenoids are phytochemical agents of diverse groups originated from squalene or related acyclic such as oleanolic, betulinic, ursolic acids (MA Domingues *et al.*, 2014). oleanolic, betulinic, ursolic acids have drawn attention with increasing number of publications due to their wide range of medicinal properties in the biological system (Silva *et al.*, 2016). Recent studies showed that these terpenoids have several important biological

activities such as anti-inflammatory, antitumoral, antioxidant effects as well as nephroprotective role (Ma *et al.*, 2014; Chan *et al.*, 2014). Oleanolic acid (OA) in particular, has been shown to contain antioxidant potency in scavenging free radicals and possesses both anti-inflammatory, hepatoprotection properties in liver injury (Zhao *et al.*, 2013; Wang *et al.*, 2011). Because the liver is a key organ in different metabolic pathways and activities of the body; OA serve to protect the liver from toxicity. OA as being used to improve the action of insulin in models induced with diabetes, promote glucose utilization as well as inhibiting gluconeogenesis (Wang *et al.*, 2013a).

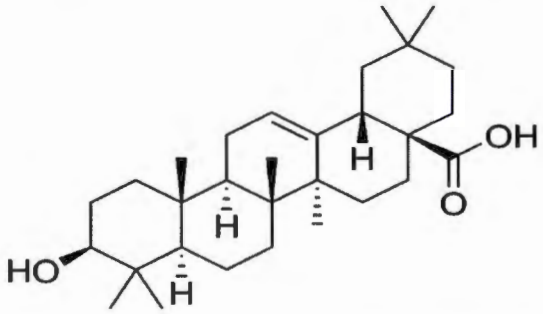

Figure 2.5: Chemical structure of oleanolic acid (Cao *et al.*, 2018)

According to Tang *et al.* (2007) vitamin D and calcium (ca) which are often used as supplements for the management of healthy bones in adults. Although, the potential increase in the risk of developing cardiovascular complication due to the use of Ca supplement becomes a concern. OA serve as bone protective triterpenoid compound identified in most dietary plants and medicinal herbs about 1620 of them with no side effect (Fukushima *et al.*, 2011). In terms of anti-inflammatory and anti-cancer potentials, OA demonstrated anti-inflammatory effect by inhibiting hyperpermeability in the expression of cell adhesion molecules (CAMs) and migration of leukocyte, thus showing its therapeutic ability in vascular inflammatory diseases (Lee *et al.*, 2013). The report of the biological activities of OA was also shown in the in vivo antioxidant assessing model by ameliorating oxidative injury induced by tert-Butyl hydroperoxide (tBHP) through increasing the generation of antioxidant (glutathione) and the expression of key antioxidant enzymes mediated by nuclear factorerythroid 2 p45-related factor 2 (Nrf2) (Wang *et al.*, 2010).

3.0 CHAPTER THREE

3.1 MATERIALS AND METHODS

3.1 Ethical clearance

The study was conducted after obtaining animal ethical clearance at the North-West University, Mafikeng campus and University of the Witwatersrand, Johannesburg (Ethics clearance number: 2014/D/47), South Africa.

3.2 Animal housing

Each dam and its pups were housed in the same Perspex cages with stainless steel mesh lids. Shredded paper was used as bedding and changed twice a week. The dams did not receive any experimental treatment but were provided with normal commercial rat chow (Epol®, Johannesburg, South Africa) and water *ad libitum* throughout the suckling period. During the 7-day experimental period, dams were allowed to freely nurse until euthanasia of the rat pups on postnatal day (PD) 14. The room temperature was maintained at 25 ± 2°C. The dams and their pups were placed on a 12-h light and dark cycle (with lights switched on at 07:00 am). There was adequate ventilation of the room at all time.

3.3 Study design

Twenty-four (N=24) Sprague Dawley pups' rats were used for this study. The Sprague Dawley rats were randomly divided into four (4) different treatment groups each with (N=6) male and female rats. The experimental treatments were started on postnatal day (PD) 7 and ended on PD 14. The neonatal rats were euthanized by an intraperitoneal injection of sodium pentobarbital (200 mg/kg body mass; Euthanaze ®, Centaur Laboratories, Johannesburg, SA). For ethical reasons and because of their age, the pups were not fasted prior to termination.

3.3.1 Treatments of rats from day 1– 14

Day 1 – 6: Acclimatization of the pups.

Day 7 – Day 14: The pups were administered with a high fructose diet and oleanolic acid. All treatments were administered once daily, for seven days (PD7 to PD13), at a volume of 10 mℓ/kg body mass via orogastric gavage. Dietary fructose was administered neonatally to induce neonatal program for the development of metabolic abnormalities in the later stage of life. All pups were observed for 20 minutes for unusual behavioural changes and clinical signs of toxicity of the treatments throughout the course of the experimental treatments. All the rats in all the treatment groups were sacrificed at day 14.

Table 3.1. Treatment groups of the suckling male and female Sprague Dawley pups rats

Groups	Treatments
A	Control – distilled water: rats received dH_2O (10 mℓ/kg) for 1 week
B	Oleanolic acid (60 mg/kg): rats received oleanolic acid (10 mℓ/kg) for 1 week
C	High fructose solution (25% w/v): rats received high fructose solution (25%) for 1week
D	High fructose solution (25% w/v) + Oleanolic acid (60 mg/kg): rats received high fructose solution (25%) and oleanolic acid (10 mℓ/kg) for 1 week

The study was conducted using the skeletal muscles (triceps muscle) of the rats by following the established protocols in the laboratory and that of manufacturer's protocol for respective methods.

3.4 Terminal procedures, collection and preparation of samples

3.4.1 Collection of samples

At the end of the 7-day treatment period, (PD14) experimental treatment were stopped and the pups were euthanized by an intraperitoneal injection of sodium pentobarbital (200 mg/kg body mass;

Euthanaze ®, Centaur Laboratories, Johannesburg, South Africa). For ethical reasons and because of their age, the pups rats were not fasted prior to termination. The skeletal muscles (triceps muscle) were dissected out, snap frozen in liquid nitrogen, and then stored at -80°C freezer in a cryovial tubes for further experiments.

3.4.2 Preparation of samples homogenate

Muscular tissue (triceps) samples from -80°C freezer were collected and kept in ice. They were weighed (gram) and suspended in a 15 ml falcon tube. Homogenate was done in (10% w/v) freshly prepared phosphate buffer (50 mM Na-Pi, 0.5% (v/v) Triton x-100, pH 7.5). The samples were homogenized with a homogenizer (stuart homogenizer SHM1/382 made in USA). Homogenate was centrifuged for 30 minutes 10,000 rpm at 4°C (Thermo scientific SL 8R GO Cat N0. 75007224, Germany) and supernatant were collected in a labelled cryovial tubes and stored in a freezer at -80°C for further analysis.

3.5 Assay for antioxidant enzymes

3.5.1 Catalase assay

Catalase enzyme activity was determined using the method described by Sinha (1972). Hundred microliters (100 µl) of homogenized sample was measured into labelled falcon tube for both test/control sample. Hundred microliters (100 µl) of distilled H_2O was measured as standard/blank. To each of control test/blank, 1000 µl distilled H_2O was added and 1000 µl of hydrogen peroxide (H_2O_2 (65 mM) in 50 mmol/L sodium, potassium phosphate buffer) was added to each of sample test/standard. Mixtures were vortexed and incubated at 37°C for 3 minutes. One thousand microliter (1000 µl) dichromate/acetic acid was added to each of the tubes. This was then taken to water bath and kept at 100°C for 10 minutes after which it was left to cool under tap water, then centrifuged to remove precipitated protein (2500 *g* for 5 min). The change in absorbance were recorded at 570 nm against the reagent blank using spectrophotometry (Thermo scientific™ Multiskan™ GO Cat N0. N13133/2015 Model, Ratastie 2, FI-01620 Vantaa, Finland).

The equation below was used to determine catalase enzyme activity:

Catalase Activity of test kU= 2.303/t*[log S^O/S-M] *Vt/Vs

where; t = time.

S^o = Absorbance of standard tube.

S = Absorbance of test tube.

M = Absorbance of control test (correction factor).

Vt = Total volume of reagents in test tube.

Vs = Volume of serum.

3.5.2 Superoxide dismutase assay

The superoxide dismutase (SOD) activity was determined using SOD assay kits-WST. This was done per SOD kits instruction protocol (Sigma-Aldrich® 3050 Spruce street, St. Louis, MO 63103 USA). Twenty microliters (20 µl) of sample solution was measured into each of sample and blank 2 well, and 20 µl of distilled water (H_2O) was measured into blank 1 and blank 3 well. Two hundred microliters (200 µl) of WST working solution was added to each well and it was mixed. Twenty microliters (20 µl) of dilution buffer was added to blank 2 and blank 3 and 20 µl of enzyme working solution was added to both sample and blank 1. These mixtures were then shaken thoroughly to mix. The microplate reader (Thermo scientific™ Multiskan™ GO Cat N0. N13133/2015 Model, Ratastie 2, FI-01620 Vantaa, Finland) was incubated at 37°C for 2 minutes and the absorbance were recorded at 450 nm.

The equation below was used to determine the SOD activity

SOD (inhibition rate %) = {[(Ablank 1 -Ablank 3) – (Asample – Ablank 2)] / (Ablank 1 – Ablank 3)} * 100

3.5.3 Glutathione peroxidase assay

Glutathione peroxidase activity was determined as per Rotruck *et al.* (1973). Standard working solution of glutathione (GSH) was prepared (0.0307 g GSH dissolved in 100 ml (0.2 M) EDTA solution (pH 8)) in 25 µM, 50 µM, 100 µM, 150 µM, and 250 µM concentration. Phosphate buffer

(K_2HPO_4 and KH_2PO_4; 100 mM (pH 7.4)) of 500 µl was measured into different labelled falcon tubes, 100 µl of sodium azide ($NaNO_3$; 10 mM) was measured into it, followed by the addition of 100 µl hydrogen peroxide (H_2O_2; 2.5 mM). Five hundred microliters of homogenized sample was added; followed by the addition of 600 µl of distilled H_2O. The whole reaction mixture was incubated at 37°C for 3 minutes after which 500 µl of Trichloro acetic acid (TCA, 10% 2 g of TCA dissolved in 20 ml H_2O) was added and thereafter centrifuged at 3000 rpm for 5 minutes. To 100 µl of each of the supernatants/standards, 200 µl of K_2HPO_4 and 100 µl of DTNB (5'-5'-dithiobis-(2-dinitrobenzoic acid) was added and the absorbance was read at 412 nm with the use of spectrophotometry (Thermo scientific™ Multiskan™ GO Cat N0. N13133/2015 Model, Ratastie 2, FI-01620 Vantaa, Finland).

Glutathione peroxidase activity was observed by plotting the standard curve and the concentration of the remaining GSH was extrapolated from the curve.

GSH consumed = 245.34 – GSH remaining

Glutathione = GSH consumed/ mg protein. Result was expressed as µM/mg protein

3.6 Gene expression of the antioxidant enzymes

3.6.1 Extraction of RNA from tissue (triceps muscle)

Extraction of RNA was determined as per Hongbao *et al.* (2008). Triceps muscle samples (200 mg) were crushed and weighed into falcon tube. The samples were kept on dry ice until homogenization procedure was performed. One ml (1ml) of Trizol was added and homogenized on ice. Chloroform (200 µl) was added to the homogenate and vortexed vigorously. It was then incubated on ice for 15 minutes. The homogenate was then centrifuged at 12000 units of times gravity (× *g*) for 15 minutes at 4°C for phase separation. The aqueous phase was transferred into a falcon tube. Thereafter, RNA was precipitated with 0.5 ml isopropanol by gentle mix. It was then incubated for a further 10 minutes on ice; and centrifuged for 10 minutes at 12000 units of times gravity (× *g*) at 4°C. Supernatant was discarded leaving pellet. One ml (1ml) of 70% ethanol was added to pellet and was

gently socked with pipette to washed pellet. This was then centrifuged at 7500 units of times gravity (× *g*) for 10 minutes at 4°C and the supernatant was discarded. The residue were air dried and RNA pellet was dissolved in an appropriate volume of RNase free water.

3.6.2 RNA integrity

This was determined using the thermo Fisher scientific protocol modified from "Current Protocols in Molecular Biology", Section 4.9 (Ausubel et al., eds.). Agarose gel (1%) was made to assess the integrity of the RNA extracted from the muscular tissue (triceps). To an empty glass bottle, 1.5 g of agarose was weighed and 150 ml Tris base, acetic acid and EDTA (TAE) buffer was added. These mixture were shaken thoroughly and heated for 60 seconds to dissolve the agarose. Thereafter, the reagent was cooled to 50^0C and 1.5 µl of Ethidium bromide was added with proper mixing. The melted agarose gel solution was poured into a casting tray sealed at both ends with comb placed in it. It was then allowed to cool until gel solidified. Gel was then transfered into the electrophoresis chamber. Four microliters (4 µl) of sample RNA plus 6 µl of bromophenol loading dye were mixed and loaded on the gel wells and TAE buffer was poured into the chamber so that it was 2-3 mm over the gel. The gel was run at 80V for 60 mins, and after the run the gel was placed in a UV box and photographed.

3.6.3 First-strand cDNA synthesis

The synthesis of cDNA was done using Applied Biosystems Superscript VILO cDNA Synthesis Kit Product information sheet (2015). To labelled falcon's tubes, 4 µl 5X VILO reaction mix, 2 µl 10X Superscript enzyme mix, were added followed by the addition of 1.92 µl, 1.34 µl, 1.07 µl and 1.1 µl of control, oleanolic acid (OA) , high fructose solution (HF) and OA+HF RNA to each tube. These were then made up to 20 µl with DEPC-treated water. Reaction mixture were gently mixed and incubated at 25^0C for 10 minutes. Thereafter, the tube contents were further incubated at 42^0C for 60 minutes and the reaction was terminated by incubation at 85^0C for 5 minutes.

3.6.4 Real-time quantitative PCR assay

The quantitative PCR gene expression was performed based on Applied Biosystems Thermo Fisher Scientific protocol PowerUp™ SYBR Green Master Mix (2015). The cDNA (2 µl) was used for PCR amplification. Specific primers belonging to targeted gene regions (β-actin, SOD2, CAT, and GPx) were designed to amplify and determine the expression level of targeted genes. A PCR reaction volume was prepared containing (5 µl PowerUp™ SYBR Green Master Mix (2X), 1 µl each of forward/reverse primers, 2 µl cDNA template plus 1 µl RNase-free water and the total reaction volume equated to 10 µl). The reagents were mixed thoroughly and centrifuged briefly to spin down the contents and eliminate any air bubbles. An appropriate volume of each reaction made was transferred to each well of an optical plate. The plate was sealed with an optical adhesive cover and the reaction plate was then placed in the PCR thermocycler that was programmed to the following conditions: UDG activation (2 min at 50°C), initial denaturation (2 min at 95°C); 40 cycles of denaturation (15s at 95°C); 40 cycles annealing and extension (1 min at 60°C).

Table 3.2: List of primers used for quantitative RT-PCR analysis

Gene	Primer sequence (5′-3′)	Product size (bp)
SOD2	Forward – GTGAACAACCTGAACGCCAC	808
	Reverse – CCTACAGGCCCCCAAAACAT	
CAT	Forward – CGGGCCTGGCCGATG	853
	Reverse – GCCATTCATGTGCCGATGTC	
GPx	Forward – CAGTCCACCGTGTATGCCTT	344
	Reverse – GTAAAGAGCGGGTGAGCCTT	
β-actin	Forward – AGATGGTTGCTGCATCTGCT	425
	Reverse – AGGAAAAACTTCATCATATTCCTGC	

3.7 Antioxidant capacity assay

3.7.1 Ferric reducing antioxidant power (FRAP)

The FRAP assay was carried out as described by Benzie and Strain (1996). The FRAP reagent was a mixture of 30 ml acetate buffer (pH 3.4), 3 ml tripyridyl triazine (TPTZ), 3 ml $FeCl_3$ and 6.6 ml distilled water (dH_2O). A hundred microliters (100 µl) homogenized sample was mixed with 200 µl FRAP reagent in a micro plate reader. The mixture was incubated for 30 minutes at room temperature and the absorbance was read at wavelength of 539 nm using a spectrophotometer. Ascorbic acid (AA) was used as the standard and the results were expressed as µmolAAE/ml sample.

3.7.2 Trolox equivalent antioxidant capacity (TEAC)

TEAC assay was analysed using the principle of 2,2-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging activity as described by (Ou *et al.* (2001)). The ABTS was prepared by mixing 88 µl $K_2S_2O_8$, and 5 ml ABTS and was left overnight before use. Twenty-five microliters (25 µl) of the sample/standard was mixed with 200 µl ABTS solution and incubated for 30 minutes at room temperature. The absorbance of the mixture was then read at 734 nm using spectrophotometer. Trolox was used as the standard and the results were expressed as µmol TE/ml.

3.8 Oxidative stress biomarkers

3.8.1 Lipid peroxidation assay

Tissue malondialdehyde (MDA) concentration was measured spectrophotometrically as thiobarbituric reactive substance by the method of Buege and Aust (1978). Aliquots mixture of 20 µl sample/standard (MDA) mixed with 100 µl of 15% (w/v) trichloroacetic acid and 100 µl of 0.375% (w/v) thiobarbituric acid. This reaction mixture was heated at 100°C for 15 minutes and cooled to room temperature before centrifuged at 3,000 g for 5 min and absorbance was measured at 535 nm. Results were expressed as nmolMDA/ηg protein.

3.8.2 Nitrite assay

Nitrite concentration was measured by the griess reaction method (Yang *et al.*, 2014). Briefly, 100 µl of griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamide in 5% phosphoric acid) was mixed with 50 µl of homogenized sample and incubated for 10 min at room temperature, and absorbance was read at 540 nm using a microplate reader. The result was expressed as µM.

3.9 Assay for inflammatory biomarkers

The levels of inflammatory biomarkers in the samples such as tumour necrosis factor-alpha (TNF-alpha), monocyte chemotactic protein-1 (MCP-1), vascular endothelial growth factor (VEGF), and interleukin (IL-1β, IL-4, IL-5, IL-6 and IL-10) were measured in the triceps muscle using Bio-Plex Pro magnetic bead-based assays (Bio-Rad Laboratories, Hercules, USA) on the Bio-Plex platform (Bio-Rad). Following previous optimization, samples were evaluated undiluted in a blinded manner. Samples were reacted with a mixture of fluorescent polystyrene beads bound with specific anticytokine primary antibodies, resulting in binding of the cytokines to the beads with the corresponding antibody. The biotinylated anticytokine secondary antibodies were then added and allowed to bind to the cytokine-bead complex followed by the addition of fluorescent phycoerythrin-conjugated streptavidin. All analytes levels in the quality control reagents of the kits were within the expected ranges. The standard curve for all the analytes ranged from 3 to 12000 pg/mL. Bio-Plex Manager software, version 6.0, was used for bead acquisition and analysis.

3.10 Statistical analysis

Data were expressed as the means ± standard deviations (S.D). Significant differences between mean values of different groups were determined by one-way analysis of variance (ANOVA) with Graph Prism 6 (GraphPad Software Inc., San Diego, USA). $P<0.05$ was accepted as significant.

4.0 CHAPTER FOUR

4.1 RESULTS

Table 4.1 shows the activities of antioxidant enzymes in the different groups. There was a significant increase ($p<0.05$) by 2.5-fold in catalase activity in OA group (1.37 ± 0.01 kU/L) as compared to HF treated group and insignificant with control, OA+HF while HF group was the least with (0.53 ± 0.01 kU/L). The control and OA+HF groups showed a significant increase by 2-fold in catalase enzyme activity with 1.08 ± 0.01 kU/L and 1.03 ± 0.04 kU/L respectively as compared to the HF group. The glutathione peroxidase (GPx) enzyme exhibited a higher degree level of activity within the OA treated group (627.5 ± 45.7 µM/mg protein) as compared to other groups while, HF fed groups also showed a rising shift of activity (555.3 ± 29.3 µM/mg protein), but was insignificant as compared to the control and OA+HF; although it showed increased activity in the HF above the control (308.5 ± 19.4 µM/mg protein) and the OA+HF (533.2 ± 31.1 µM/mg protein). The results showed no significant difference in the inhibition of SOD activity, although the HF has the highest percentage inhibition rate (96.55%) as compared to control, OA and OA+HF groups with 78.22%, 93.40% and 94.12% respectively.

Table 4.1: The activities of antioxidant enzymes in the groups

Groups	Parameters		
	GPx (μM/mg protein) Tissue	SOD Activity (%) Tissue Inhibition rate	CAT activity (kU/L) in Tissue
Control	308.5 ± 19.4	78.22 ± 3.60	1.08 ± 0.01 *
OA	627.5 ± 45.7	93.40 ± 0.85	1.37 ± 0.05 **
HF	555.3 ± 29.3	96.55 ± 0.39	0.53 ± 0.01
OA+HF	533.2 ± 31.1	94.12 ± 1.23	1.03 ± 0.04 *

Control: Distilled H_2O, OA: Oleanolic acid, HF: high-fructose solution, OA+HF: Oleanolic acid/high fructose. GPx= Glutathione peroxidase, SOD= Superoxide dismutase, CAT= Catalase, ($p<0.05$) level of significance. (**) indicate significant differences across the groups.

4.2 Gene expression of antioxidant enzymes

4.2.1 Superoxide dismutase activity

The results (Figure 4.1) showed the expression of superoxide dismutase (SOD) genes in all the groups. The SOD gene showed higher expression in the OA group as compared to the control, HF and OA+HF groups. The graph below shows a significant increase by 6.5-fold between the OA group and HF group ($p<0.05$), and indicated a 0.3-fold increase when compared with that of the control; statistically it was insignificant to OA+HF. The control group as well as the group fed with OA+HF showed an increased value of expression by 6-fold when compared to the HF treated group ($p < 0.05$). The HF treated group being administered with high fructose solution exhibited the least expression with a significant decrease by 6-fold to OA, 4-fold to control and 4.8-fold to OA+HF treated groups. Both OA and OA+HF groups were shown to be significantly different as compared to the control group ($p < 0.05$) although, there was no difference between the OA treated group and OA+HF group respectively.

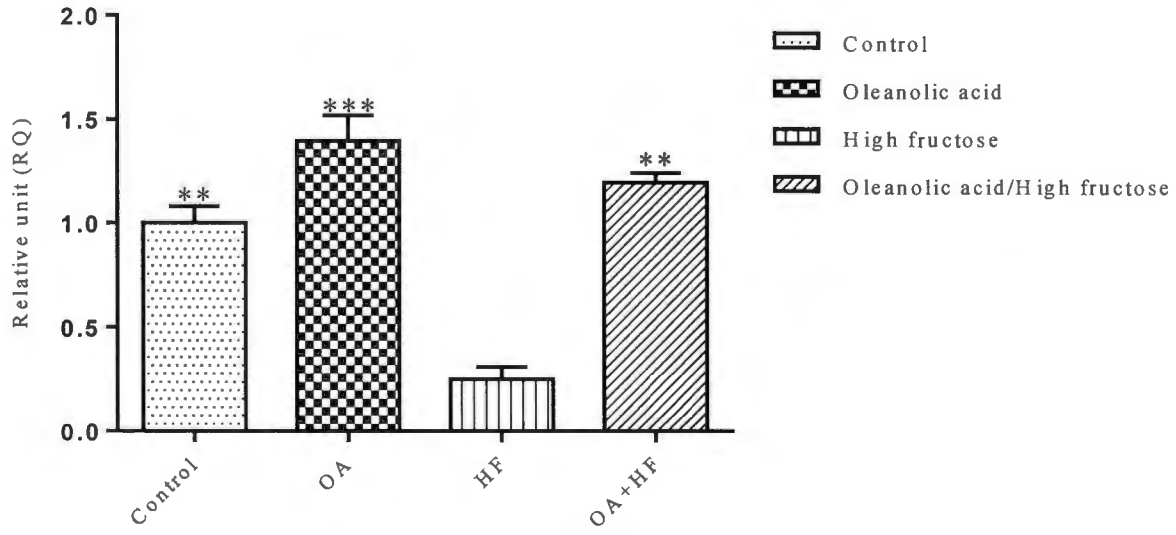

Figure 4.1: Expression of superoxide dismutase (SOD) gene with β-actin as an internal control. Control: (Distilled H2O), OA: oleanolic acid, HF: High fructose solution, OA+HF: oleanolic acid/high fructose, ($p<0.05$) level of significance. (**) indicate significant differences between the groups.

4.2.2 Catalase activity

Results in Figure 4.2 show the *in vivo* assessment of catalase gene expression in the muscle (triceps) tissue. The results demonstrated a significant difference ($p < 0.05$) between the OA group and HF treated group at ($p < 0.05$) by 10-fold increase in the gene expression while there was no significant difference statistically between the control and OA+HF groups. Furthermore, results in Figure 4.2 also reveals a remarkable significant difference by 6-fold between the control and the HF fed group ($p < 0.05$), but there was no significant difference between the control and OA. Similarly, a trend of difference was observed between the control and OA+HF by one-fold but statistically insignificant ($p < 0.05$). The expression of catalase in the HF treated group significantly decreased in when compared to all groups; although statistically, the result indicated an insignificant different ($p < 0.05$) with OA+HF.

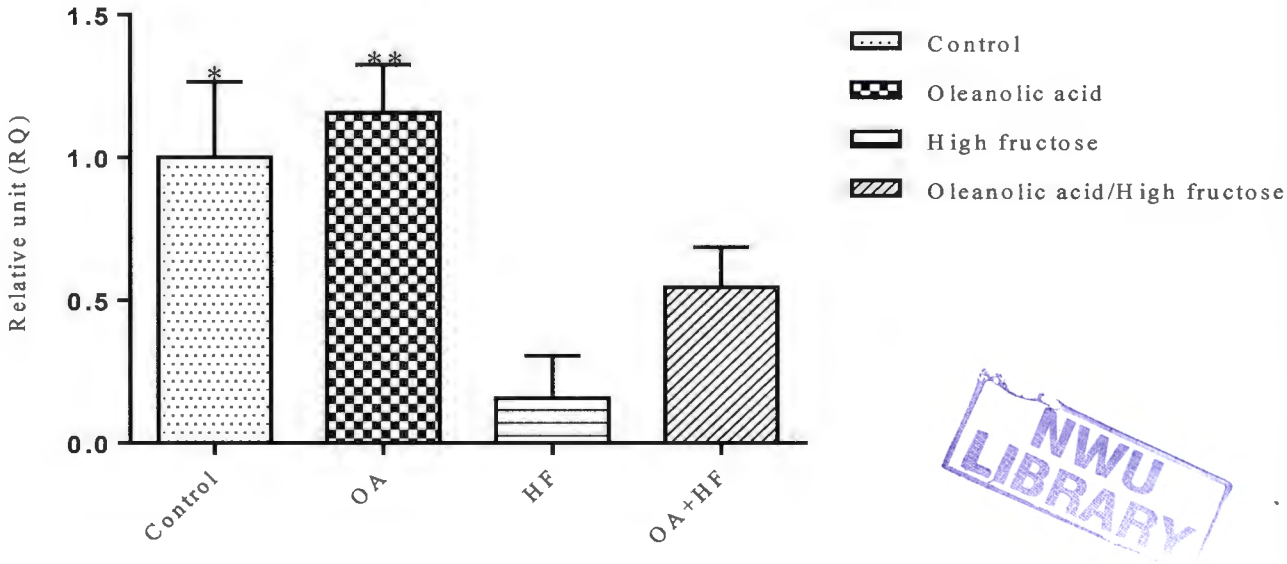

Figure 4.2: Expression of catalase (CAT) gene with β-actin as an internal control. Control: (Distilled H2O), OA: oleanolic acid, HF: High fructose solution, OA+HF: oleanolic acid/high fructose, ($p<0.05$) level of significance. (**) indicate significant differences between the groups.

4.2.3 Glutathione peroxidase activity

The GPx gene expression in Figure 4.3 below shows a remarkable significant increase at ($p<0.05$) in groups fed with oleanolic acid (OA, OA+HF) as compared to the group fed with HF . OA as indicated in the graph (Figure 4.3) showed a significant increase ($p < 0.05$) by 5-fold to the HF treated group but was statistically insignificant to the control and OA+HF. However, the control group showed an increase in GPx expression by 3-fold as compared to HF, but was insignificant at ($p < 0.05$) statistically as well as with the OA+HF. The OA+HF group exhibited a significant increase ($p < 0.05$) by 5-fold as to the HF group. The group fed with high fructose solution showed the least gene expression of GPx. However, control, OA and OA+HF groups showed no significant difference.

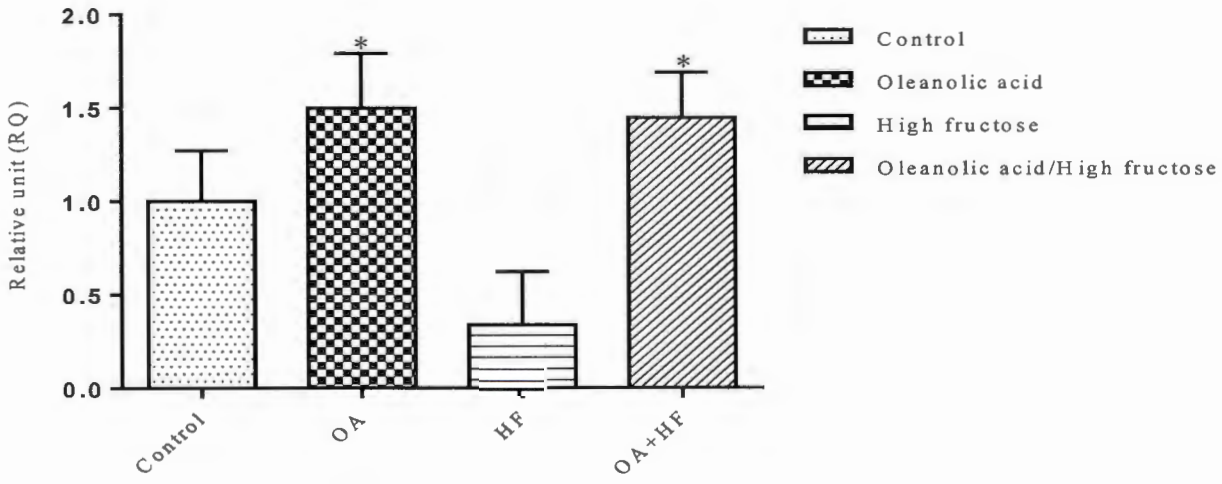

Figure 4.3: Expression of glutathione peroxidase (GPx) gene with β-actin as an internal control. Control: (Distilled H2O), OA: oleanolic acid, HF: High fructose solution, OA+HF: oleanolic acid/high fructose, ($p<0.05$) level of significance. (**) indicate significant differences between the groups.

4.4 RNA Integrity

RNA integrity was assessed using a 1% agarose gel. The lace G1, G2, G3 and G4 as shown in (figure 3.1) are the RNA bands of the Control, OA, HF and OA+HF groups respectively with 18s size (1.9 kilobase) and 28s size (4.7 kilobase) bands confirming the intactness of extracted RNA. M is the Ambion millennium markers hundred base pair (100 bp).

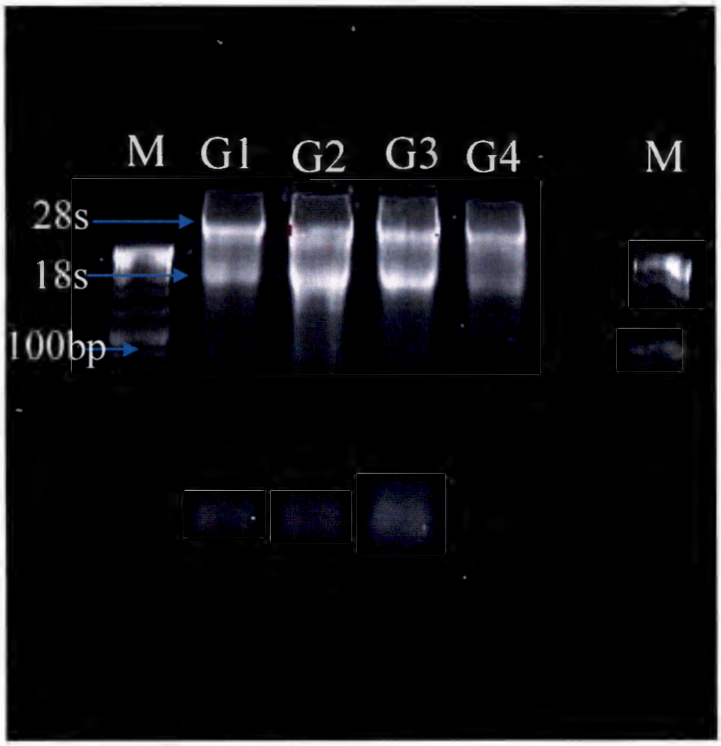

Figure 4.4: Agarose gel showing RNA integrity using gel.

4.5 Antioxidant capacity

In Table 4.2, results shows that the Ferric reducing antioxidant power (FRAP) had no significant difference ($p<0.05$) across all the groups. The results showed a non-significant increase in TEAC in HF group $p>0.05$ with the value of 469.3 μmolTE/g as compared to the control, OA and OA+HF groups having 356.4 μmolTE/g, 392.8 μmolTE/g and 393.9 μmolTE/g.

Table 4.2: The antioxidant capacity in the experimental groups

Group	Parameters	
	TEAC (μM/ml)	FRAP (μM/ml)
Control	356.4 ± 13.4	36.98 ± 5.24
OA	392.8 ± 5.79	30.16 ± 2.54
HF	469.3 ± 11.9	30.22 ± 1.68
OA+HF	393.9 ± 12.4	36.95 ± 4.56

Control: normal diet, OA: oleanolic acid, HF: high-fructose solution, OA+HF: Oleanolic acid/high fructose. TEAC= Trolox Equivalent Antioxidant Capacity, FRAP= Ferric Reducing Antioxidant Power, ($p<0.05$) level of significance. (**) indicate significant differences across the groups.

4.6 Oxidative stress biomarkers

4.6.1 Lipid peroxidation

The results in Figure 4.5 show that the levels of malondialdehyde (MDA) within each groups were significantly different ($p<0.05$) with the HF group having the highest concentration (50.5 nmolMDA/ng protein) as compared to the control (18.3 nmolMDA/ng protein). Statistically, the concentration showed a significant increase by 2.5-fold to the control but when compared to OA, OA+HF malondialdehyde concentration shows an increase although it was not significant statistically. OA+HF (48.5 nmolMDA/ng protein) showed remarkable significant difference by 2-fold with the control and the difference was significant. However, the result showed that OA (34.4nmolMDA/ng protein) and the control indicate an insignificant difference, although when compared to OA+HF group it was insignificant respectively.

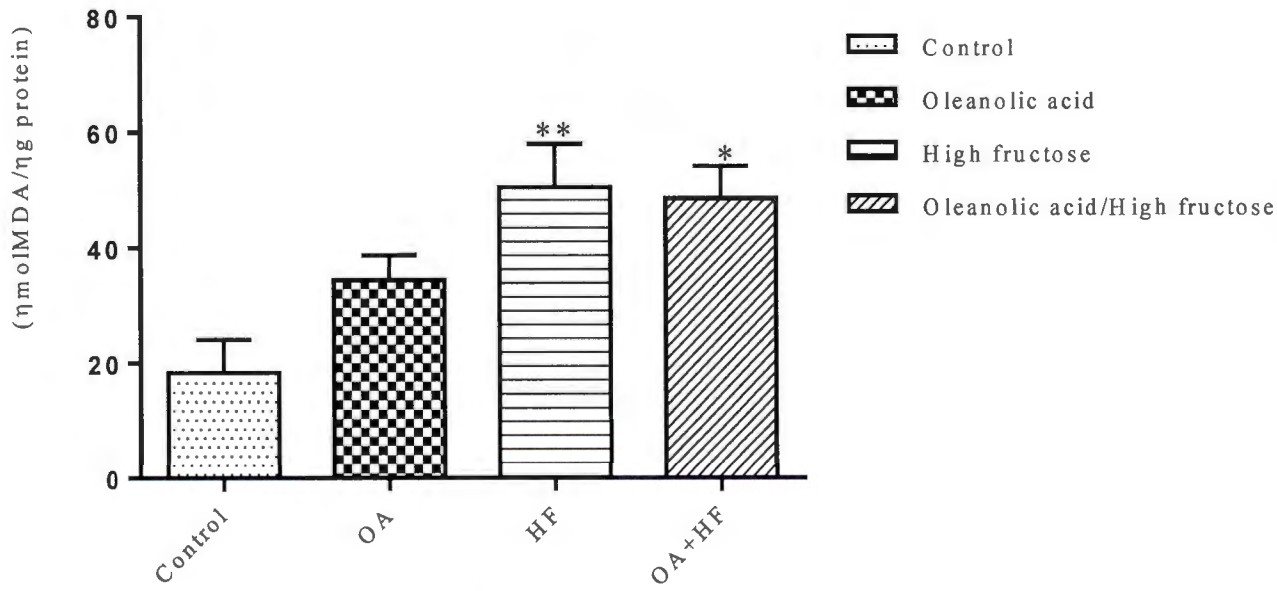

Figure 4.5: The level of lipid peroxidation. Control: (Distilled H2O); OA: oleanolic acid, HF: High fructose solution; OA+HF: Oleanolic acid/high fructose, ($p<0.05$) level of significance. (**) indicate significant differences between the groups.

4.6.2 Total glutathione

Results in Figure 4.6 shows total glutathione across all groups with a remarkable significant difference in OA (4142.8 mM/mg protein) at ($p<0.05$) by 12-fold as compared to HF (320.4 mM/mg protein) treated group. OA showed significant increase by 2-fold to control. Total glutathione was statistically insignificant when compared to the control and OA+HF groups. The Control (1690.1 mM/mg protein) group also showed a high concentration of total glutathione as compared to HF treated group at ($p<0.05$) by 5-fold. However, when compared to the groups supplemented with oleanolic acid (OA, OA+HF), the result showed no difference statistically. The OA+HF (3330.5 mM/mg protein) group showed a significant increase by 2-fold with that of the control group ($p<0.05$) but no statistical difference between the control and OA group respectively. Although it produced a significant increase ($p<0.05$) by 10-fold as compared to HF treated group.

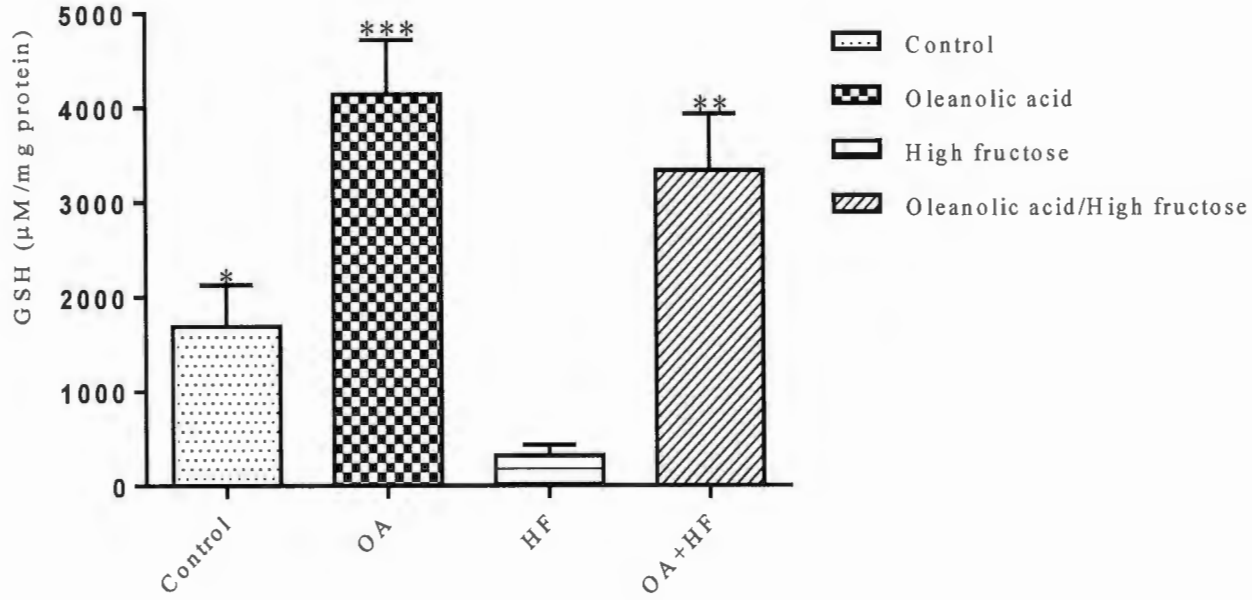

Figure 4.6: Total glutathione (GSH) level. Control: distilled H2O; OA: oleanolic acid, HF: High fructose solution; OA+HF: oleanolic acid/high fructose, ($p<0.05$) level of significance. (**) indicate significant differences between the groups.

4.6.3 Nitrite concentration

The nitrite level concentration across all groups showed no significant difference at ($p<0.05$). Although, from the graph below (Figure 4.7), the control (30.6 μM) and HF (29.8 μM) shows slight increase as to the groups supplemented with oleanolic acid OA (27.2 μM) and OA+HF (27.3 μM), but was insignificantly different statistically.

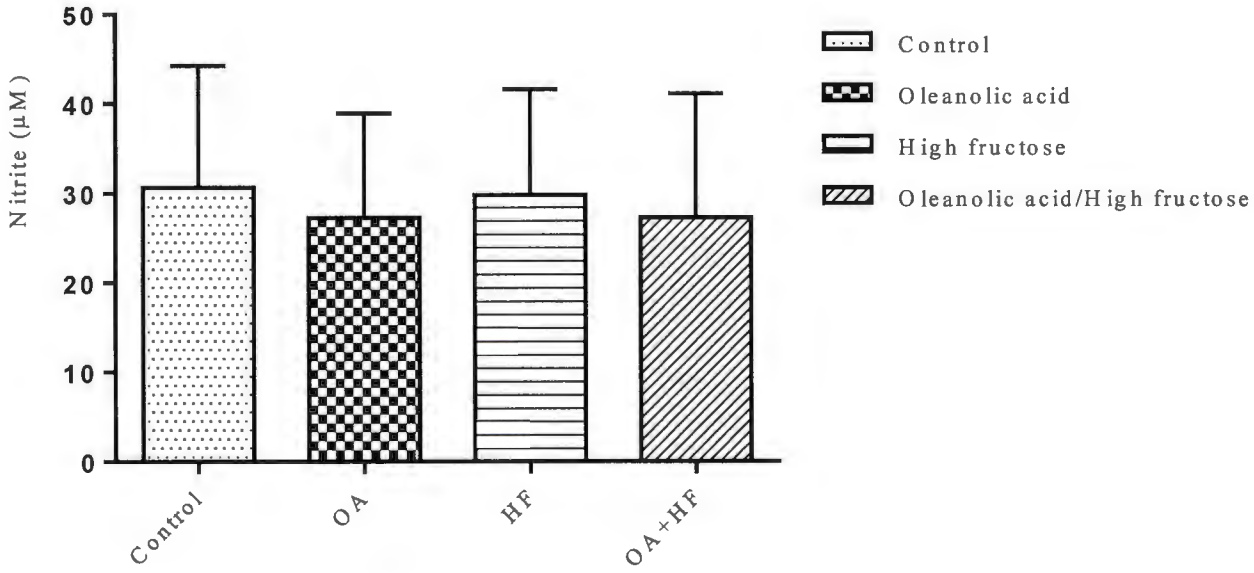

Figure 4.7: Nitrite level concentration. Control: distilled H_2O; OA: oleanolic acid, HF: High fructose solution; OA+HF: oleanolic acid/high fructose. ($p<0.05$) level of significance. (**) indicate significant differences between the groups.

4.7 Inflammatory biomarkers

4.7.1 Pro inflammatory and anti-inflammatory cytokines

Results of pro-inflammatory cytokines in Tables 4.3a and 4.3b show no statistical significant differences across all groups at ($p<0.05$). However, for each pro-inflammation cytokine (IL-1b, IL-5, IL-6, IL-12, IFN, TNF, VEGF, MCP-1), HF treated group showed a tendency to increase inflammation as compared to other groups. The anti-inflammatory biomarkers (IL-4, IL-10) in (Table 4.4) was not significant in groups supplemented with OA as compared to control and HF treated groups ($p>0.05$). Although, across all groups both OA, OA+HF groups revealed a non-significant difference statistically when compared to the control and HF groups.

Table 4.3a: Pro-inflammatory cytokines concentration in the experimental groups

Groups	Parameters			
	IL-1b (pg/ml)	IL-5 (pg/ml)	IL-6 (pg/ml)	IL-12 (pg/ml)
Control	487.1 ± 40.1	63.72 ± 2.63	606.0 ± 42.6	305.5 ± 23.2
OA	446.0 ± 35.4	61.74 ± 3.44	547.7 ± 34.5	274.0 ± 23.0
HF	667.2 ± 41.6	68.47 ± 3.37	914.1 ± 68.0	366.9 ± 22.8
OA/HF	384.6 ± 33.9	63.14 ± 3.36	754.6 ± 72.1	280.5 ± 22.3

Pro-inflammatory concentration level was measured as a marker. Control: distilled H_2O; OA: oleanolic acid, HF: High fructose solution; OA/HF: oleanolic acid/high fructose. ($p<0.05$) level of significance. (**) indicate significant differences across the groups.

Table 4.3b: Pro-inflammatory cytokines concentration in the experimental groups

Groups	Parameters			
	IFN-g (pg/ml)	TNF-a (pg/ml)	VEGF (pg/ml)	MCP-1 (pg/ml)
Control	366.3 ± 26.7	191.1 ± 14.1	201.3 ± 11.7	700.2 ± 44.6
OA	317.2 ± 23.0	152.5 ± 7.06	193.5 ± 8.35	642.9 ± 43.0
HF	373.3 ± 25.5	207.3 ± 17.8	245.6 ± 15.3	877.1 ± 78.2
OA/HF	332.9 ± 23.1	195.7 ± 19.3	156.5 ± 5.73	741.0 ± 60.0

Anti-inflammatory concentration level was measured as a marker. Control: distilled H_2O; OA: oleanolic acid, HF: High fructose solution; OA/HF: oleanolic acid/high fructose. ($p<0.05$) level of significance. (**) indicate significant differences across the groups.

4.7.2 Anti-inflammatory cytokines

Table 4.4: Anti-inflammatory cytokines concentration in the experimental groups

Groups	Parameters	
	IL-4 (pg/ml)	IL-10 (pg/ml)
Control	49.53 ± 4.43	691.8 ± 41.8
OA	51.34 ± 5.63	756.1 ± 54.3
HF	45.33 ± 3.24	650.9 ± 37.7
OA/HF	54.48 ± 6.03	597.3 ± 45.3

Anti-inflammatory concentration level was measured as a marker. Control: distilled H_2O; OA: oleanolic acid, HF: High fructose solution; OA+HF: oleanolic acid/high fructose diet. ($p<0.05$) level of significance. (**) indicate significant differences across the groups.

5.0 CHAPTER FIVE

5.1 Discussions

This study was designed to investigate the potential protective effect of neonatal (7 days) oral administration of oleanolic acid (OA) against fructose-induced oxidative damage in skeletal muscle, development of negative health complications in suckling male and female pups. One of the fundamental causes of developing oxidative stress in our society and the increase of mortality rate, is the uncontrolled continuous ingestion of high fat diet and intake of larger amount of carbohydrate sugar (Zhang *et al.*, 2017). These also play a vital role in causing oxidative stress and induces damages on basic macro molecules such as DNA, protein, lipids (Tangvarasittichai, 2015). The influx of industries with the aim to produce dietary sugary foods with deleterious effect to the life span of an individual cannot be over emphasized. In this study, we showed that short-term neonatal administration of OA protected against fructose-induced oxidative damage and cause no precocious development in suckling pups. The prophylactic use of OA in the fight against oxidative stress prevents oxidative damage with no side effects on pups.

In this study, the activities of antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPx1) and catalase (CAT) was determined. This study demonstrated that neonatal oral administration of high fructose downregulated their activities (table 4.1), which was prevented by neonatal administration of OA. SOD, GPx and CAT are key antioxidant enzymes that constitute cellular defence mechanism and protects the body against reactive oxygen specie (ROS) (Tian *et al.*, 2017). The presence of these enzymes is important in the defence against free radical induced injury, oxidative damage and DNA depletion (Arita *et al.*, 2006; Salvi *et al.*, 2007). Excessive fructose consumption negatively affects the cellular anti-oxidant capacity through its effects on the generation of ROS which causes a decrease in the levels and activities of GPx, CAT and SOD2 (Abdel-Kawi *et al.*, 2016). A previous study in an insulin resistant adult rat model of genetic hypertension showed that OA possesses anti-oxidant activities (Sloboda *et al.*, 2014). In an *in vitro* anti-oxidant activity-assessing model, OA acted as a free radical-scavenger through direct chemical

reactions and a biological molecule, which may have enhanced the anti-oxidant defences (Wang *et al.*, 2010). The protective effect of OA against fructose-induced oxidative damage observed in this study could be attributed to the anti-oxidant effect of OA reported previously in animal models (Gao *et al.*, 2009; Wang *et al.*, 2013b) It has also been reported that OA regulated the activity of GPx and SOD in the lungs in a system threatened by oxidative stress (Peng et al., 2017). These findings suggest that OA improves antioxidant defence mechanisms which affirm with our findings. Pups were exposed to high intake of fructose and at the same time administed with OA via orogastric gavage. After two weeks of experimention, the results showed a significant increase in CAT activity in OA group as compared to control and OA+HF groups. Supplementation with OA improved CAT activity in OA+HF group as compared to control group. The activity of CAT was significantly decreased in the HF group when compared with the control group and increased in OA+HF group. The HF group showed significant increase in rate of inhibition of SOD activity as compared to control, OA and OA+HF groups. However, there was no significant difference between the control group and OA+HF and OA groups. GPx activity showed an increased in OA group, but when compared to control and OA+HF groups remained unchanged. Although, it was statistically insignificant when compared across all groups.

In the current study, the gene expression of anti-oxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx1) was determined. We showed that neonatal oral administration of fructose downregulated the expression of CAT, SOD2 and GPx1 by 25%, which was prevented by neonatal administration of OA. Previous studies in which expression of genes for anti-oxidant enzymes was assessed, showed that the anti-oxidant capacity in fructose-fed rats was decreased (Girard *et al.*, 2006). Fructose increases the influx of triglycerides into hepatocytes causing overproduction of ROS through the β-oxidation of free fatty acids (Furukawa *et al.*, 2017). The elevation of pro-oxidant species causes damage to cellular membranes and DNA (Thannickal and Fanburg, 2000). The observed downregulation of anti-oxidant enzyme genes in fructose-fed rats in

this study could be attributed to the effect of fructose on the generation of ROS through mitochondrial β-oxidation (Botezelli *et al.*, 2012).

This present study also showed a significant decrease in the expression of CAT, SOD and GPx genes in HF fed group as compared to control, OA+HF, and OA groups. The OA treated group showed significant increase in gene expression as compared to control and HF groups. OA+HF showed significant increase in GPx gene expression when compared with HF group and insignificantly different as compared to the control group. The low expression of genes in the HF group could be associated to the high intake of the fructose diet and gene expression was improved following OA supplementation.

The capacity of antioxidants in the muscular tissue was analysed by quantifying its antioxidant capacity for TEAC. TEAC indicated an increased in the antioxidant capacity in the HF group as compared to control, OA and OA+HF groups. FRAP values across all the groups were non-significantly different. This significant increase in antioxidant capacity as shown in HF fed group can be as a result of the ability of the body to fight against the attack of the free radicals.

The results showed a reduction in the concentration of GSH in HF group as compared to OA, control and OA+HF groups. In OA+HF group, the depletion observed in the HF group was followed with a significant increase in GSH when supplemented with OA. The GSH in OA+HF group was significantly higher when compared to the control group. It can be deduced from this study that HF diet depleted GSH content in the rats while supplementation with OA increased the GSH content. The results also showed a significant increase in lipid peroxidation in the HF group than control, OA and OA+HF treated groups. This indicates that the level of lipid peroxidation; a biomarker of oxidative stress expressed as the degree of accumulation of malondialdehyde (MDA) was significantly increased presumably due to oxidative stress. The nitrite concentration in all the groups was insignificant.

The results showed no significant differences in pro-inflammation biomarkers. Similarly, anti-inflammatory cytokines were not significantly different within the experimental period. However, pro-inflammatory cytokines showed the tendency to increase in the HF treated group though not significant. Also, anti-inflammatory cytokines showed a non-significant increase in rats fed with oleanolic acid (OA and OA+HF groups). A similar study by Yoo *et al.* (2017) showed that rats model ingested with high-fructose diet significantly proliferate fat weight, but the serum pro-inflammatory cytokines did not change. Moreover, the short time exposure of the pups to high fructose diet in this study could also be a reason for the non-significant effects and hence, it is recommended that further investigations are being carried out to the adult stage.

5.2 Conclusion

Supplementation with oleanolic acid in pups' rats showed the tendency to attenuate fructose-induced oxidative damage by suppressing the levels of free radicals. It also resulted to the activation of antioxidant enzymes that are part of the antioxidant defence mechanism in the body. These findings provide evidence supporting the benefit of oleanolic acid as a supplement that can help in regulating and scavenging excess free radical's production to ameliorate oxidative stress. Further research should be done to the adult stage of the Sprague Dawley rat in order to assess its effectiveness.

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APPENDIX

Catalase gene (CAT)

ANOVA summary	
F	5.846
P value	0.0049
P value summary	**
Are differences among means statistically significant? (P < 0.05)	Yes
R square	0.4672

Number of families	1
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. OA	-0.1590	-0.9024 to 0.5844	No	Ns
Control vs. HFD	0.8430	0.09956 to 1.586	Yes	*
Control vs. OA/HFD	0.4550	-0.2884 to 1.198	No	Ns
OA vs. HFD	1.002	0.2586 to 1.745	Yes	**
OA vs. OA/HFD	0.6140	-0.1294 to 1.357	No	Ns
HFD vs. OA/HFD	-0.3880	-1.131 to 0.3554	No	Ns

Glutathione peroxidase gene (GPx)

ANOVA summary	
F	3.857
P value	0.0250
P value summary	*
Are differences among means statistically significant? (P < 0.05)	Yes
R square	0.3665

Number of families	1
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary

Control vs. OA	-0.5000	-1.585 to 0.5854	No	Ns
Control vs. HF	0.6630	-0.4224 to 1.748	No	Ns
Control vs. OA+HF	-0.4480	-1.533 to 0.6374	No	Ns
OA vs. HF	1.163	0.07764 to 2.248	Yes	*
OA vs. OA+HF	0.05200	-1.033 to 1.137	No	Ns
HFD vs. OA+HF	-1.111	-2.196 to -0.02564	Yes	*

Superoxide dismutase

ANOVA summary	
F	36.13
P value	< 0.0001
P value summary	****
Are differences among means statistically significant? (P < 0.05)	Yes
R square	0.8442

Number of families	1
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. OA	-0.3960	-0.7259 to -0.06610	Yes	*
Control vs. HFD	0.7510	0.4211 to 1.081	Yes	****
Control vs. OA/HFD	-0.1960	-0.5259 to 0.1339	No	Ns
OA vs. HFD	1.147	0.8171 to 1.477	Yes	****
OA vs. OA/HFD	0.2000	-0.1299 to 0.5299	No	Ns
HFD vs. OA/HFD	-0.9470	-1.277 to -0.6171	Yes	****

Oxidative stress biomarkers

Total glutathione

ANOVA summary	
F	10.41

P value	0.0002
P value summary	***
Are differences among means statistically significant? (P < 0.05)	Yes
R square	0.6096

Number of families	1
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. OA	-2453	-4545 to -360.2	Yes	*
Control vs. HF	1370	-722.8 to 3462	No	Ns
Control vs. OA+HF	-1640	-3733 to 452.1	No	Ns
OA vs. HF	3822	1730 to 5915	Yes	***
OA vs. OA+HF	812.3	-1280 to 2905	No	Ns
HF vs. OA+HF	-3010	-5102 to -917.6	Yes	**

Lipid peroxidation

ANOVA summary	
F	6.480
P value	0.0031
P value summary	**
Are differences among means statistically significant? (P < 0.05)	Yes
R square	0.4929

Number of families	1
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. OA	-16.07	-39.23 to 7.088	No	ns
Control vs. HF	-32.14	-55.30 to -8.986	Yes	**
Control vs. OA+HF	-30.15	-53.31 to -6.997	Yes	*
OA vs. HF	-16.07	-39.23 to 7.083	No	ns
OA vs. OA+HF	-14.09	-37.24 to 9.072	No	ns

HFD vs. OA+HF	1.989	-21.17 to 25.15	No	ns

Nitrite

ANOVA summary	
F	0.1088
P value	0.9540
P value summary	ns
Are differences among means statistically significant? (P < 0.05)	No
R square	0.01605

Number of families	1			
Number of comparisons per family	6			
Alpha	0.05			
Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. OA	3.365	-17.31 to 24.04	No	Ns
Control vs. HF	0.8243	-19.85 to 21.50	No	Ns
Control vs. OA+HF	3.311	-17.36 to 23.99	No	Ns
OA vs. HF	-2.541	-23.22 to 18.13	No	Ns
OA vs. OA+HF	-0.05405	-20.73 to 20.62	No	Ns
HFD vs. OA+HF	2.486	-18.19 to 23.16	No	Ns