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Effect of hypoxia on metabolic markers and

gene expression HIF-1 α in adipocytes

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By

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COMMITTEE

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Abstract

Background:

Docosahexaenoic acid (DHA; omega-3 fatty acid) has been reported to have potential anti-obesity properties. Hypoxia is a condition that results from the excessive expansion of white adipose tissue resulting in obesity-related conditions including insulin resistance, inflammation and oxidative stress.

Methods:

The objective of this study was to test the effects of DHA on the hypoxia responses (1.0 % for 24 hours) of 3T3-L1 adipocytes with a focus on oxidative stress, inflammation, and mitochondrial function, and antioxidant status. Cell viability, reactive oxygen species (ROS) and apoptosis were measured by flow cytometry. The metabolic parameters such as lactate, glycerol release, glucose uptake and adenosine triphosphate (ATP) content were measured by fluorometer. The expression of (hypoxia inducible factor 1 α; HIF-1 α) and the secretion of adipocytokines were evaluated by qPCR (quantitative polymerase chain reaction) and ELISA (enzyme linked immunosorbant assay).

Results:

Under hypoxia conditions, DHA treatment resulted in significant changes in all critical parameters of adipocyte biology including HIF-1α RNA expression (decreased by 50%), decreased lactate and glycerol release (66% and 25% respectively), and reactive oxygen species (ROS) production (decreased by 15%), while glucose uptake was decreased by 25% accompnied by decreased secretion of pro-inflammatory markers (Interleukin 6 IL-6, 31% reduction) , macrophage chemoattractant protein 1 MCP-1 (38% reduction) and leptin (14% reduction), and increased adiponectin secretion (by 45%). The exposure of adipocytes to 1% hypoxia significantly alters the transcript of hypoxia genes, and other genes involved in glucose (GLUT1, GLUT4) and lipid (PPAR-γ) metabolism, and is also linked to anaerobic metabolism with apoptosis and increased oxidative stress.

Conclusion:

Data indicate that DHA can exert potential anti-hypoxia effects by reducing the secretion of inflammatory adipocytokines, oxidative stress, lipolysis and apoptosis. This may ameliorate the deleterious effects of excess adiposity.

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ABBREVIATIONS

3T3-L1   Cell line which was originated from clonal expansion of murine Swiss 3T3 cells and contain only a single cell type

AMM   Adipocyte Maintenance Medium

ATP   Adenosine triphosphate

BAT   Brown adipose tissue

BMI   Body Mass Index

BSA   Bovine serum albumin

CCR2   Chemokine (C-C motif) receptor 2

CHD   Coronary heart disease

CVD   Cardiovascular disease

DNA   Deoxyribonucleic acid

FBS   Fetal bovine serum

GLUT1   Glucose transporter 1

GLUT4   Glucose transporter 4

HIF-1α Hypoxia-inducible factor 1-alpha

IBMX   3-isobutyl-1-methylxanthine

IL-6   Interleukin-6

IκB   Inhibitor nuclear factor

JNK   c-Jun N-terminal kinase

MCP-1   Macrophage chemoattractant protein -1

MDA   Malondialdehyde

PAI-1   Plasminogen activator inhibitor-1

PBS   Phosphate buffered saline

PPAR-γ Peroxidase proliferator-activated regulator-gamma

qRT-PCR   Quantitative Reverse Transcription PCR

RNA   Ribonucleic acid

ROS   Reactive oxygen species

T2DM   Type 2 diabetes mellitus

TBA	Thiobarbituric Acid
TNF-α	Tumor necrosis factor-alpha
WAT	White adipose tissue

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<center>**Chapter 1**</center>

1. Introduction

1.1 Background

Obesity is due to an imbalance between energy intake and consumption (expenditure). Obesity is generally related to insulin resistance, metabolic syndrome, and hyperinsulinemia, and is also associated with increased cardiovascular disease (CVD) and/or progression to type 2 diabetes mellitus (T2DM) (Kahn & Flier, 2000). Adipocytes secrete more than sixty adipokines through which adipose tissue can cross-talk with other tissues and organs. These adipokines are involved in various functions such as energy balance, glucose and lipid metabolism, insulin-sensitization, immune function, inflammation, hemostasis, and angiogenesis (Trayhurn & Wood, 2004). The production of certain adipokines is interconnected with the pathogenesis of metabolic disorders related to obesity (Trayhurn & Wood, 2004).

Increased adipocyte size up to 140-180 μm in diameter is a characteristic feature of obesity (Brook, Lloyd, & Wolf, 1972), resulting in surpassing the normal O_2 diffusion distance i.e. 100-200 μm (Brahimi-Horn & Pouyssegur, 2007). Therefore, hypertrophic adipocytes might suffer from hypoxia. The cellular adaptation of adipocytes to hypoxia is conserved by activation of hypoxia-inducible factor 1α (HIF-1α). HIF-1α is one of the specific transcription factors. HIF-1α increases during hypoxia and upregulates mRNA expression of a broad range of genes that stimulate angiogenesis, glycolysis and erythropoiesis (Semenza, 2000).

Recent studies have suggested that hypoxia upregulates the expression of many adipokine genes, including MCP-1and IL-6, PAI-1 and leptin while adiponectin expression is down-regulated (Wood, de Heredia, Wang, & Trayhurn, 2009). Also, hypoxia induces insulin resistance via HIF-

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1α action and the development of an inflammatory state related to obesity in response to hypoxia (Trayhurn & Wood, 2004).

Due to reduction in the oxidative phosphorylation, the metabolism of the cell changes to anaerobic glycolysis with lower efficiency of ATP production, placing an increased demand for glucose with increased GLUT-1 expression in adipocytes (Wang, Wood, & Trayhurn, 2007). Because of anaerobic glycolysis, lactic acid production is increased and lactate exported from the cell (Wood et al., 2009). This metabolic switch is regulated by HIF-1α as the most important intermediary of hypoxic signaling (Rich, 2003). The omega-3 fatty acid, Docosahexaenoic acid (DHA), has been found to ameliorate the inflammatory and insulin resistance effects of excess adiposity (J. J. Li, Huang, & Xie, 2008).

Based on previous fundings, I hypothesized that exposure of adipocytes to low O_2 tension (1.0 %; hypoxia) would generate a chronic inflammatory state associated with incresaed oxidative stress, accompanied by a switch of metabolism to anaerobic glycolysis, which mimics hypertrophic obesity (larger adipocyts) and insulin resistance. I further hypotheized that DHA could reverse the hypoxic changes in adipocytes.

1.2 Aims and objectives

The main objective of this dissertation was to study the metabolic and biological effects of hypoxia on a cell/tissue culture, utilizing a model of metabolically active tissue (3T3-L1 adipocytes) to assess the response to hypoxia. 3T3-L1 adipocytes exposed to 21% O_2 tension (normoxia) used as a control group for comparison. The following experiemts were conducted:

A. **Normoxic cell group [control]**

B. **Hypoxic cell group**

C. **Treatment group: DHA applied to hypoxic cell group.**

The following parameters were assessed, in the above experimental conditions:

1. Metabolic markers: glucose, and lactate released in the medium and ATP content from the cell lysate before and after exposure to hypoxia and after DHA application.

2. The expression of the inflammatory markers and adipokines such as MCP-1, IL-6, leptin and adiponectin in response to hypoxia and after DHA application.

3. The expression of the HIF-1 α and GLUT-1in response to hypoxia and after DHA application.

4. Evaluation of the influence of DHA on markers of inflammation and oxidative stress in 3T3-L1 cells after expose to hypoxia, and after DHA application.

CHAPTER 2

2. LITERATURE REVIEW

2.1. Obesity

Abnormal accumulation of excessive fat defines obesity. Clinically, obesity is defined by a body mass index (BMI; weight (kg)/[height (m)]^{2}) of 30 kg/m². Obesity results from an imbalance between energy expenditure and energy intake (first law thermodynmics). Obesity is a multifactorial disorder with strong genetic basis (Cordero, Li, & Oben, 2015).

Energy balance can be altered by several conditions including genetic and enironmental factors (Kopelman, 2000).

Since 1980, obesity prevalence has doubled. The World Health Organization (WHO) latest statistics estimated that 11% of adults are obese worldwide. According to recent data, Qatar ranked sixth in the Middle East for obesity prevalence with an estimated 33.1% of the population being affected (WHO, 2015). Obesity is a major risk factor for insulin resistance and the metabolic syndrome and chronic health disorders, such as, type 2 diabetes, and cardiovascular disease (Steinberger & Daniels, 2003).

2.2. Risk Factors for Obesity

The pathological growth of adipocytes is an outcome of the disproportion between energy consumption and energy expenditure (Ahn, Lee, Kim, Park, & Ha, 2008). The progression of the adipose tissue may be an outcome of cell hyperplasia and/or cell hypertrophy, which indicates

proliferation in the numbers and dimensions of cells, respectively (Sano et al., 2014). Recent findings sugget that obesity is associated with a chronic inflammatory state. Inflammatory factors released by adipocytes (adipokine) control carbohydrate and lipid, metabolism, and immune function. These adipokines, could be used as circulating markers of cardio-metabolic risk/disease (Rocha & Folco, 2011). Obesity increases the risk of many diseases that translates to increased mortality and morbidity. Obesity associated co-morbidities include coronary heart disease (CHD), type 2 diabetes (T2DM), hypertension, dyslipidemia, kidney disorders, and some types of cancer (Pi-Sunyer, 2002).

2.3. Adipose tissue and Adipocytes Complexity

Adipose tissue is an areolar connective tissue that regulates and maintains body temperature, attaches the skin, and shields internal organs. Adipose tissue is sub-divided into two types; brown adipose tissue (BAT) and white adipose tissue (WAT). Each tissue type has its specific features: WAT serves as the reservoir of energy, where triglycerides are stored in large lipid droplet, while BAT serves to regulate body temperature and energy expenditure (Lafontan, 2008). White and brown adipose tissues are differentially distributed. (WAT) dominates in adult humans, but brown adipose tissue (BAT) is generally found in neonates. Studies have shown that WAT is responsible for obesity and its complications such as T2DM. This is because WAT produces a greater number of adipokines (Farmer, 2008). Also, recent studies have reported that WAT is an active endocrine organ through relaesed adipookines which also have paracrine actions (Trayhurn, 2005). On the other hand, BAT cells are rich in mitochondria and their primary function is heat generation by fatty acid oxidation. Unlike WAT, triglycerides in BAT are

distributed in multiple small lipid droplets (Cannon & Nedergaard, 2004). Furthermore, studies found that dysregulation of WAT function leads to hyperglycemia and dyslipidemia, which ultimately cause insulin resistance and chronic inflammation (Trayhurn, 2005).

Adipocytes are assumed to originate from mesenchymal stem cells, but still many developmental steps of the process are unclear (Guilak et al., 2006). Nevertheless, several investigators have suggested the ability of neuroepithelial cells to form mature adipocytes (Takashima et al., 2007). Even bone marrow progenitor cells may covert into adipocytes that can be differently accumulated according to age and weight (Rodeheffer, Birsoy, & Friedman, 2008; Tang et al., 2008). The process of differentiation of the fibroblast that is similar to preadipocytes into mature lipid-laden, insulin-responsive adipocytes, is known as adipogenesis (Lefterova & Lazar, 2009). Preadipocytes have a similar morphology to fibroblasts; however, when they differentiate into mature adipocytes, they become round puffy cells with large fat droplet pushing the nucleus to the border and making the cytoplasm condensed into a thin layer (Ali, Hochfeld, Myburgh, & Pepper, 2013).

The adipogenesis process ends up with mature adipocytes that have gone through the main stages of differentiation. It starts with mesenchymal precursors becoming committed preadipocytes that undergo growth arrest until they start dividing again and clonally expand before they ultimately differentiate and become mature adipocytes (Lefterova & Lazar, 2009).

2.4. 3T3-L1 Cell Line

Many 3T3-L1 Cell Line is an established model for the understanding and evaluation of adipocyte biology (Poulos, Dodson, & Hausman, 2010). The 3T3-L1 cell is a pre-adipocytes cell line that is originated from the clonal growth of murine Swiss 3T3 cells (Green, 1974). More than five thousand articles have been published on the topics of adipogenesis and the adipocytes biochemistry over the last 30 years and have been using this cell line extensively, due to its ability to differentiate from fibroblast to complete adipocytes (Zebisch, Voigt, Wabitsch, & Brandsch, 2012). Many researchers choose to utilize 3T3-L1 cells as it aids in the recognition of important molecular indicators together with the transcription factors, adipokine secretion, gene/protein expression, and various pathways during preadipocyte differentiation (Poulos et al., 2010). Numerous different procedures can be utilized to persuade the differentiation process to adipocytes from pre-adipocytes, but the most common agents used are 3-isobutyl-1-methylxanthine (IBMX), insulin and dexamethasone (Russell & Ho, 1976). Pre-adipocytes start with low lipid content but with gowth, they begin to accumulate lipids. They grow in size and number over the period of differentiation that takes three to four days after induction (Zebisch et al., 2012).

2.5. Inflammation and Obesity

Adipocytes have a dynamic role as endocrine cells because they release numerous bio-active materials called "adipocytokines" or ""adipokines", which have a direct influence on food consumption, the vascular microenvironment, sensitivity to insulin and energy metabolism (Kuroyanagi, 2008). Recently, investigators have concentrated on the concept that obesity could be a source of inflammatory factors, which enhance insulin resistance and create a chronic low-grade systematic inflammation (O. Y. Kim et al., 2012). Adipokines and bioactive peptides, including, adiponectin, plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor-alpha (TNF-α), leptin, resistin, interleukin-6 (IL-6), macrophage chemoattractant protein-1 (MCP-1), were are released from adipocytes (Sung et al., 2011). The metabolic condition gets worse with the release of excessive amounts of pro-inflammatory cytokines/adipokines by adipocytes (paracrine and endocrine fashion) such as PAI-1, TNF-α, resistin, IL-6, , MCP-1 etc ; which inhibit the insulin action on target tissues such as muscle, liver and islet cells inducing insulin resistance (McCall, 2010). The inflammation that is produced due to obesity could be mediated by multi-cellular stresses, such as hypoxia, endoplasmic reticulum, and oxidative stress (Attie, 2009).

2.6. Adipokines

2.6.a. Leptin

Zhang et al. (1994) described leptin as a 16-kDa hormone that is produced primarily by the ob gene and is released by the adipocytes. Its primary purpose remains controlling of body weight through the leptin receptor (Flier, 1995). Leptin circulates in the plasma and its levels are correlated with total body adiposity (Frederich et al., 1995). In the fed state, the levels of leptin rise and act through the hypothalamus. This leads leads to loss of appetite. Other actions include the stimulation of thyroid-mediated thermogenesis and fatty acid oxidation. During fasting, the process reverses. A study by Farooqi and colleauges (Farooqi et al., 2002) linked the deficiency of leptin to the increase in appetite and body weight in humans and mice. This process can, however, be reversed by recombinant leptin treatment. Considering obesity in humans, leptin concentrations are directly proportional to the body size (Considine et al., 1996), and the body weight reacts modestly to recombinant leptin (Heymsfield et al., 1999) due to leptin resistance (Flier, 2004).

Other functions of leptin include roles in hematopoiesis, homeostasis, angiogenesis (Sierra-Honigmann et al., 1998) and reproduction (Ahima et al., 1996). In the year 1998, Lord et al. showed the importance of leptin in macrophages and monocytes where it increases the phagocytic functions and in the production of proinflammatory markers (Mancuso et al., 2002). Leptin also affects chemotaxis in polymorphonuclear cells of healthy subjects. In NK cells, leptin takes part in cell development, activation, and cytotoxicity.

2.6.b. Adiponectin

Adiponectin is a circulatinf protein hormone with plasma levels ranging between 3-30 mg/mL in lean subjects. Adipnoectin levels are lower in obese individuals (Arita et al., 2012). Adiponectin forms three major complexes: high molecular weight (HMW), hexamer, and trimer forms. The HMW form is the bioactive form mostly in the vascular endothelial cells (Okamoto, Kihara, Funahashi, Matsuzawa, & Libby, 2006). Adiponectin has a bioactive product that comprises the C1q-like globular domain. This domain can circulate at a lower concentration in plasma (Fruebis et al., 2001). The receptors of adiponectin are AdipoR1 and AdipoR2 present in different tissues to mediate the adiponectin action. Abnormal receptor structure and subsequent function results in glucose intolerance and insulin resistance (Yamauchi et al., 2007).

Studies have linked adiponectin to antidiabetic features (Okamoto et al., 2006). Adiponectin gene knockout deficient mice clear plasma fatty acids at a lower rate than wild type mice and have insulin resistance (Maeda et al., 2002). The insulin sensitivity in these animals improved through adiponectin replacement through adenovirus (Maeda et al., 2002). In a different study, adiponectin overexpression in ob/ob mice resulted in improvement in glucose tolerance and a reduction in triglyceride levels. (J. Y. Kim et al., 2007). Other scientists have argued for various features of adiponectin including anti-atherogenic and anti-inflammatory effects. The inflammatory effects have been linked to atherogenesis, involving endothelial cells and macrophages (Ouchi et al., 1999). Monocyte attachment to the endothelium in culture has been attenuated due to the adiponectin physiological levels. This attenuation affected adhesion molecules through reducing TNF-α-induced expression (Ouchi et al., 1999). The mechanism of anti-inflammation displayed by the adiponectin is not fully understood. Pretreatment of

adiponectin present in human macrophages slowed down the process of phosphorylation of inhibitor nuclear factor (IκB), c-Jun N-terminal kinase (JNK) and p38 MAPK induced by the activation of phosphorylation of transcription 3, that was induced by IL-6 as well as TNF-α (Folco, Rocha, Lopez-Ilasaca, & Libby, 2009). Such studies are the basis of the hypothesis that adiponectin induces activation of the anti-inflammatory process (Folco et al., 2009).

In a meta-analysis, involving approximately 14,500 individuals higher concentrations of adiponectin were associated with a lower risk of T2DM (S. Li, Shin, Ding, & van Dam, 2009). A recent comparative study of insulin-sensitive and insulin-resistant parameters in obese people clearly proved that the levels of adiponectin in the circulation and infiltration of macrophages in adipose tissue were the strongest predictors of insulin sensitivity (Kloting et al., 2010). There is a very close association between T2DM and adiponectin malfunction (Waki et al., 2003) that is reduced by genetic mutation. This knowledge supports a primary role for adiponectin as a defence mechanism for metabolic diseases.

There is an inverse relationship between levels of adiponectin plasma levels and hypertension incidence (Adamczak et al., 2003) dyslipidemia, and CVD (Ouchi et al., 1999). CVD patients have hypoadiponectinemia (Ouchi et al., 1999). Men having coronary artery diseases and T2DM have been shown to have lower levels of adiponectin compared to T2DM patients without CVD (Hotta et al., 2000).

There is also a two-fold increase in CVD prevalence in men with lower concentrations of plasma adiponectin, after control for several potential confounders (Kumada et al., 2003). According to

other work, higher concentrations of the plasma adiponectin are directly associated with a reduced risk of myocardial interactions in healthy individulas and reduced risk of CVD in those suffering from T2DM (Schulze et al., 2005). Some studies have failed to observe a relationship between hypoadiponectinemia and the risk of future CVD (Lindsay et al., 2005), while others did not establish significant links between plasma adiponectin and CVD (Sattar et al., 2006). Some secondary studies have examined the impact of adiponectin on CVD (Cavusoglu et al., 2006). Further studies are necessary to shed more light on adiponectin as a marker of CVD and whether increasing adiponectin levels has a beneficial effect.

2.6.c. Interleukin-6 (IL-6)

IL-6 is a key cytokine. It is under the negative control of glucocorticoids and is positively regulated by catecholamines. Therefore, it stimulates the hypothalamic-pituitary-adrenal axis (Papanicolaou, Wilder, Manolagas, & Chrousos, 1998). It is also a strong inducer of the acute-phase reaction that is present in higher levels of stress and the presence of an inflammatory or infectious disease. The role that is played by IL6 in mice is not clear yet. Mice with IL-6 deficiency frequently develop obesity that is partly overturned with IL-6 (Wallenius et al., 2002). Alternatively, mice exposed to IL-6 over a long period will develop a hepatic insulin resistance (Klover, Zimmers, Koniaris, & Mooney, 2003). In both cases, the IL-6 plays a modulatory role. IL-6 is contained in several immune cell types. Adipose tissue contains high levels of IL-6, with the subcutaneous adipose tissue layer releasing approximately 25% of circulating IL-6 in humans (Mohamed-Ali, Pinkney, & Coppack, 1998). IL-6 induces hepatic C reactive protein (CRP) (Yudkin, Stehouwer, Emeis, & Coppack, 1999). Both IL-6 and CRP are associated with insulin

resistance, hyperglycemia, and T2DM (Sandler, Bendtzen, Eizirik, & Welsh, 1990). A high association between CRP levels and baseline IL-6 and the risk for T2DM has been observed (Pradhan, Manson, Rifai, Buring, & Ridker, 2001).

2.6.d. Macrophage chemoattractant protein-1 (MCP-1)

Recruitment of macrophages to inflammation sites is mediated by chemokine (C-C motif) receptor 2 (CCR2) and MCP-1. In diet-induced obese mice, MCP-1 resulted in a reduction in the accumulation of macrophages in adipose tissue (Kanda et al., 2006). Hepatic steatosis and reduced resistance to insulin was exhibited MCP-1 knockout animals, signifying that the systemic and local metabolic effects of obesity were contributions from the influx of adipose tissue macrophage (Kanda et al., 2006). Alternatively, in the obesity-induced inflammatory process, the function of the MCP-1/CCR2 is not understood completely as not all researchers have suggested that the deficiency of MCP-1 influences the accumulation of macrophage in adipose tissue (Kirk, Sagawa, McDonald, O'Brien, & Heinecke, 2008).

2.7. Hypoxia Theory

The reason as to why the production and the release of various adipokines related to inflammation rises in obese people leading to the development of tissue inflammation is still unclear. Increased number and size of adipocytes results in peripheral adipocytes that are hypoxic and this hypoxia may drive inflammation in adipose tissue. The ratio of the cardiac output and the level up to which the blood can flow is not increased in spite of the significant accumulation of the mass of adipose tissue in obesity (Trayhurn & Wood, 2004). In lean subjects, although the

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flow of blood rises postprandial in adipose tissue this does not occur in obese subject (Virtanen et al., 2002).

Adipocytes of large size, that is about 150-200 μm in diameter (Skurk, Alberti-Huber, Herder, & Hauner, 2007), are larger as compared to the standard O_2 diffusion distance of approximately 100-200 μm (Brahimi-Horn & Pouyssegur, 2007). It has been highlighted that for some situations and some tissues, the value of PO_2 can be very low or close to zero at a distance of 100 μm with respect to the vasculature (Brahimi-Horn & Pouyssegur, 2007).

In response to hypoxia, the induction and progression of the inflammation is observed in adipose tissue of an obese person. This inflammation is associated directly with the impact of low PO_2 levels. Endoplasmic reticulum stress mechanism is enhanced with the progression of inflammation (Gregor & Hotamisligil, 2007) and oxidative stress mechanisms. The importance of the role played by gut microbiota in the inflammatory response is becoming increasingly evident (Cani et al., 2008).

2.8.   Hypoxia gene expression in adipocytes:

2.8.a. Transcription Factor HIF-α

In overweight animals, the observation that adipose tissue is hypoxic has attracted numerous studies that have sought to examine oxygen's effect on the biological functions of adipocytes and its underlying mechanisms. Initial studies concentrated on specific genes associated with angiogenesis and inflammation in mice, as well as human subjects. The studies sought to answer whether "hypoxia-induced alterations in adipokine production are mediated by hypoxia-inducible

factor 1 (HIF-1)" (Semenza, 2003). HIF-1 refers to a heterodimer that is made up of subunits α and β that express that HIF-1β are insensitive to oxygen (Semenza, 2003). The transcribing factor is done under abiotic conditions through stabilization of the HIF-1α subunit.

2.9.Hypoxia general metabolism

Hypoxia in adipose tissue can possibly control glucose utilization linked to the glycolytic pathways. A well-recognized response to lower amount of oxygen is a reflection of the switch to anaerobic metabolism. PCR arrays or microarrays have shown the expression of the genes (glycolytic pathway) that are to be upregulated in adipocytes as a result of hypoxia. The glycolytic pathways genes include glucose-6-phosphate isomerase (GPI), phosphofructokinase, platelet (PFKP) and hexokinase 2 (HK2).

Studies depict an increased presence of lysates of 3T3-LI that are represented by the proteome analysis. Transport of the increased hexose can be seen through the high rates of glucose utilization. GLUT1 is a facilitative glucose transporter that is key to basal glucose uptake. GLUT1 it is highly sensitive to hypoxia (Semenza, 2003) and has been used as a determinant of response by cells to low pO_2. In response to the low oxygen condition, GLUT1 gene expression increases in adipocytes for mice as well in humans (Wang et al., 2007; Ye, Gao, Yin, & He, 2007). GLUT1 does not handle the facilitative role alone. There has been evidence of transcripts presence for GLUT12, GLUT10, GLUT5, and GLUT3 in human adipocytes (Wood, Hunter, & Trayhurn, 2003; Wood, Wang, Lorente-Cebrian, & Trayhurn, 2007). Of the variety of transporters, GLUT5, GLUT3, and GLUT1 each have been shown to be upregulated in response to hypoxia in terms of gene expression. However, in the case of GLUT12, GLUT10, GLUT4, no

response has been observed in gene expression to low pO_2, at least over periods of up to 24 hours (Wood et al., 2007). The changes in GLUT3 and GLUT5 do not reflect the effect of the protein transporter's amount. A study has ascertained that longer duration exposure to low levels of pO_2 ensured an increased GLUT1 withholds for a longer periods of time of up to 48 hours before a fall in the expression is observed (Pérez de Heredia F, 2009). Reflected at the protein level the observation would suggest a compromise in the insulin sensitivity of GLUT4 because of the long term exposure to hypoxia. More amount of GLUT1 protein indicates that the basal glucose consummation's capacity has been improved as well as other conditions. It has been demonstrated by using 2-deoxy-D-glucose in the functional studies that, on exposure to hypoxia, the intake or absorption of glucose is indeed increased significantly. In the existence of cytochalasin B, this raised uptake of glucose is blocked, demonstrating that it is mediated by a transporter (Regazzetti et al., 2009; Wood et al., 2007) In adipocytes of transgenic mice having selective HIF-1β knockdown in the adipose tissue, the uptake of 2-deoxy-D-glucose exhibits a decline and this results in combination with reduction in gene expression of GLUT4 and GLUT1 (Lee, Gesta, Boucher, Wang, & Kahn, 2011). However, in 3T3-LI adipocytes, the HIF-1β knockdown has been observed to regroup in the GLUT4 and GLUT1 protein (Lee et al., 2011). An in-depth analysis of the importance of the HIF-1 system shows the importance of the lesson as far as modulating glucose in the adipose tissue is concerned.

As well-recognized in tumors, improved catabolism and glucose uptake through the glycolytic pathway would expected to raise the production of lactate (Gatenby & Gillies, 2004). Under hypoxic conditions, there is significant increase in lactate production and release by adipocytes of murine and human origin (Lolmede, Durand de Saint Front, Galitzky, Lafontan, & Bouloumie,

2003; Perez de Heredia, Wood, & Trayhurn, 2010), and the adipose tissues (white) of obese mice showed increased level of lactate (Hosogai et al., 2007).

2.10. Docosahexaenoic acid (DHA)

In recent decades, various research groups have been interested in studying long chain polyunsaturated fatty acids n-3 (LC-PUFA n-3) because of their observed beneficial effects on human, as well as animal health. The most beneficial results of LC-PUFA n-3 are diminution of dementia (Alzheimer's disease), cancer and cardiovascular disease (Bucher, Hengstler, Schindler, & Meier, 2002; Carrie, Abellan Van Kan, Rolland, Gillette-Guyonnet, & Vellas, 2009). It has been reported that DHA has a defensive role in various chronic inflammatory conditions, such as; psoriasis, asthma, rheumatoid arthritis and Crohn's disease (Oliver, McGillicuddy, Phillips, Toomey, & Roche, 2010). The PUFA n-3, plays a role as an effective participant in the anti-inflammatory actions, with the ability to reduce production of cytokines and eicosanoids (pro-inflammatory) (Calder, 2006). Furthermore, LCPUFA n-3 are used as substrates for the synthesis of lipid mediators, for example, anti-inflammatory resolvins and protectins (Serhan & Petasis, 2011).

CHAPTER 3

3.1. Material and Methods

Reagents and Consumables: the following Table (1) shows the list of the reagents, media, kits and consumables.

Table 3.1: Reagent List

#	Items	Manufactures	Reference #
1	Cryopreserved 3T3-L1 preadipocytes cells (Passage 14) .	Zen Bio Company, (Chapel Hill-Nelson Blvd., Suite 104, PO Box 13888/ Research Triangle Park, NC 27709, USA).	Cat# SP-L1-F
2	3T3-L1 Preadipocyte Media	Zen Bio Company, (USA).	Cat# PM-1-L-1
3	3T3-L1 Adipocyte Differentiation Medium	Zen Bio Company, (USA).	Cat# DM-2-L-1
4	3T3-L1 Adipocyte Maintenance Media	Zen Bio Company, (USA).	Cat# AM-1-L-1
5	3T3-L1 Basal Medium	Zen Bio Company, (USA).	Cat # BM-1-L1
6	Cryopreservation Medium for 3T3-L1 cell	Zen Bio Company, (USA).	Cat# FM-1-L-1-100
7	Trypsin/ EDTA (TE) solution (1X)	Gibco (Life Technologies, UK).	REF# R-001-100
8	Phosphate Buffered Saline (PBS) pH 7.4 (1X)	Gibco (Life Technologies,, UK).	Catalog #10010023
9	TRIzol Reagent Ambion RNA	Life Technologies USA	REF #15596026
10	Chloroform HPLC grade	Fisher Scientific	Code: C/4966/15
11	RNase free water	PreAnalytiX/ Qiagen, # 1057099 Hilden, Germany.	Cat# 79254
12	High Capacity RNA-to-cDNA kit	Applied Biosystems	P/N 4387406
13	TaqMan Gene Expression Master Mix	Applied Biosystems	P/N 4369016
14	TaqMan® Gene Expression Assays (Mouse PPARγ primer)	Life Technologies USA	Mm01184322_m1

15	TaqMan® Gene Expression Assays (Mouse Actb primer)	Life Technologies USA	Mm00607939_s1
16	TaqMan® Gene Expression Assays (Mouse GluT-1 primer)	Life Technologies USA	Mm00600697_m1
17	TaqMan® Gene Expression Assays (Mouse Hif 1a primer)	Life Technologies USA	Mm00468869_m1
18	PrimeTime⌐® Mini qPCR Assay,,Mm.PT.58.13819524,HIF-2 ALPHA Probe 5'-/56-FAM/ACC AGA GCC /ZEN/GTT TTT GAG AGT CAG G/3IABkFQ/-3' Primer1 5'-GAC ACG TCT TTG CTC TTC TTC-3' Primer2 5'-GAC TTC ACT CAT CCT TGC GA-3'	IDT Integrated DNA Technologies USA	Ref# 68681817
19	PrimeTime⌐® Mini qPCR Assay,,Mm.PT.58.9683859,GLUT 4 Probe,, 5'- /56-FAM/TGG AAA CCC /ZEN/GAC GGC ATC TTG T/3IABkFQ/-3' Primer 1 5'-GAG AAT ACA GCT AGG ACC AGT G-3' Primer 2,,5'-TCT TAT TGC AGC GCC TGA G-3'	IDT Integrated DNA Technologies USA	Ref# 68681821
20	PrimeTime⌐® Mini qPCR Assay,,Mm.PT.58.32860004,ANT 2 PrimeTime Probe,5'-/56-FAM/TCA CGG CAG /ZEN/ATA AGC AAT ACA AGG GC/3IABkFQ/-3' Primer1,5'-GAT ACG AAC CAC GCA GTC TAT G-3' Primer 2,5'-GCA GCC ATC TCC AAG ACA G-3'	IDT Integrated DNA Technologies USA	Ref# 68681825
21	Tali® Apoptosis Kit - Annexin V Alexa Fluor® 488 &Propidium Iodide	Life Technologies USA	Cat# A10788
22	Tali Reagent for	Life Technologies USA	Cat# C10443
23	Lipid Peroxidation (MDA) Assay Kit (Colorimetric/Fluorometric)	Abcam , Cambridge, USA	Cat#(ab118970)
24	Carboxy-H2DCFDA (general oxidative stress indicator)	Life Technologies USA	Cat# C-400
25	3T3-L1 Lipolysis Assay KIT (REAGENTS ONLY) Combo-Free Fatty acid and glycerol	Zen Bio Company, USA	Cat# LIP-3-NC-L1

	release		
26	Adiponectin Mouse ELISA	BioVendorInc	Cat# RD293023100R
27	Leptin Mouse/Rat Elisa	BioVendorInc, Int	Cat# RD291001200R
28	Interleukin-6 Mouse ELISA	BioVendorInc.Int	Cat# RAF071R
29	Monocyte Chemotactic Protein-1 Mouse ELISA	BioVendorInc.Int	Cat# RAF080R
30	ATP Assay Kit (Colorimetric/Fluorometric)	Abcam , Cambridge, USA	Cat# (ab83355)
31	Oil Red O Stain powder	Sigma-Aldrich Co. LLC St. Louis, USA	CAS Number 1320-06-5

Reagents

Cells were purchased from Zen Bio Company, (Chapel Hill-Nelson Blvd., Suite 104, PO Box13888/ Research Triangle Park, NC 27709, USA). Cryopreserved 3T3-L1 preadipocytes were received in 2ml vial containing 0.5 million cells of passage 15, Cat# SP-L1-F, and media were purchased alongside the cells from the same company (Zen Bio). The media were: 3T3-L1Preadipocyte Media, Cat# PM-1-L-1 (contains 4.5g/L D-glucose, equals ~25 mM); 3T3-L1Adipocyte Maintenance Media, Cat# AM-1-L-1; 3T3-L1 Adipocyte Maintenance basal Media without serum, Cat# AM-1-L-1-DF; 3T3-L1 Adipocyte Differentiation Medium, Cat# DM-2-L-1; and Cryopreservation Medium for 3T3-L1 cells, Cat# FM-1-L-1-100. All media except Preadipocyte Media, Cat# PM-1-L-1, contained 3.15g/L D-glucose, (equals ~17.5 mM). 3T3-L1 Lipolysis Assay KIT (REAGENTS ONLY) Combo-Free Fatty acid and glycerol release was also obtained from Zen Bio Company.

Other materials and reagents used in this study were: Trypsin/ EDTA (TE) solution (1X), REF# R-001-100, was from Gibco (Life Technologies, UK). Phosphate Buffered Saline (PBS) pH 7.4 (1X), was from Gibco (Life Technologies, UK). TRIzol Reagent Ambion RNA [REF

#15596026] was purchased from Life Technologies Grand Island, NY 14072, USA. Chloroform HPLC grade, Code: C/4966/15, was purchased from Fisher Scientific. High Capacity RNA-to-cDNA kit was purchased from Applied Biosystems P/N 4387406. TaqMan Gene Expression Master Mix (P/N 4369016); Pre-Developed TaqMan Assay Reagents, Mouse Actb (20X) (Ref# Mm00607939_s1); TaqMan® Gene Expression Assays, Ref# Mm01184322_m1 (Mouse PPARγ primer); TaqMan® Gene Expression Assays, Ref# Mm00468869_m1 (Mouse Hif 1a primer), and TaqMan® Gene Expression Assays, Ref# Mm00600697_m1 (Mouse GluT-1 primer) were all purchased from Applied Biosystems Foster, USA.

PrimeTimeᴶ® Mini qPCR Assay,,Mm.PT.58.13819524,HIF-2 ALPHA Probe 5'-/56-FAM/ACC AGA GCC /ZEN/GTT TTT GAG AGT CAG G/3IABkFQ/-3' Primer1 5'-GAC ACG TCT TTG CTC TTC TTC-3', Ref# 68681817; Primer2 5'-GAC TTC ACT CAT CCT TGC GA-3'; PrimeTimeᴶ® Mini qPCR Assay,,Mm.PT.58.9683859,GLUT 4 Probe,, 5'- /56-FAM/TGG AAA CCC /ZEN/GAC GGC ATC TTG T/3IABkFQ/-3' Primer 1 5'-GAG AAT ACA GCT AGG ACC AGT G-3' Primer 2,,5'-TCT TAT TGC AGC GCC TGA G-3', Ref# 68681821 and PrimeTimeᴶ® Mini qPCR Assay,,Mm.PT.58.32860004,ANT 2 PrimeTime Probe,5'-/56-FAM/TCA CGG CAG /ZEN/ATA AGC AAT ACA AGG GC/3IABkFQ/-3' Primer1,5'-GAT ACG AAC CAC GCA GTC TAT G-3' Primer 2,5'-GCA GCC ATC TCC AAG ACA G-3', Ref# 68681825 were all purchased from IDT Integrated DNA Technologies USA.

In addition Adiponectin Mouse ELISA (Ref# RD293023100R), Leptin Mouse/Rat Elisa (Ref# RD291001200R), Interleukin-6 Mouse ELISA (Ref# RAF071R) and Monocyte Chemotactic Protein-1 Mouse ELISA (Ref# RAF080R) were all purchased from BioVendor. Inc ,

International. Lipid Peroxidation (MDA) Assay Kit (Ref# ab118970) and ATP Assay Kit (ab83355) were all purchased from Abcam, Cambridge, USA.

All other materials used in this study were of high quality and purchased from Sigma (St. Louis, MO, USA).

3.2. Biosafety Approvals

Qatar university institutional bio-safety committee approved this study (approval number: QU-IBC 3/13-14, refer to Appendix I.
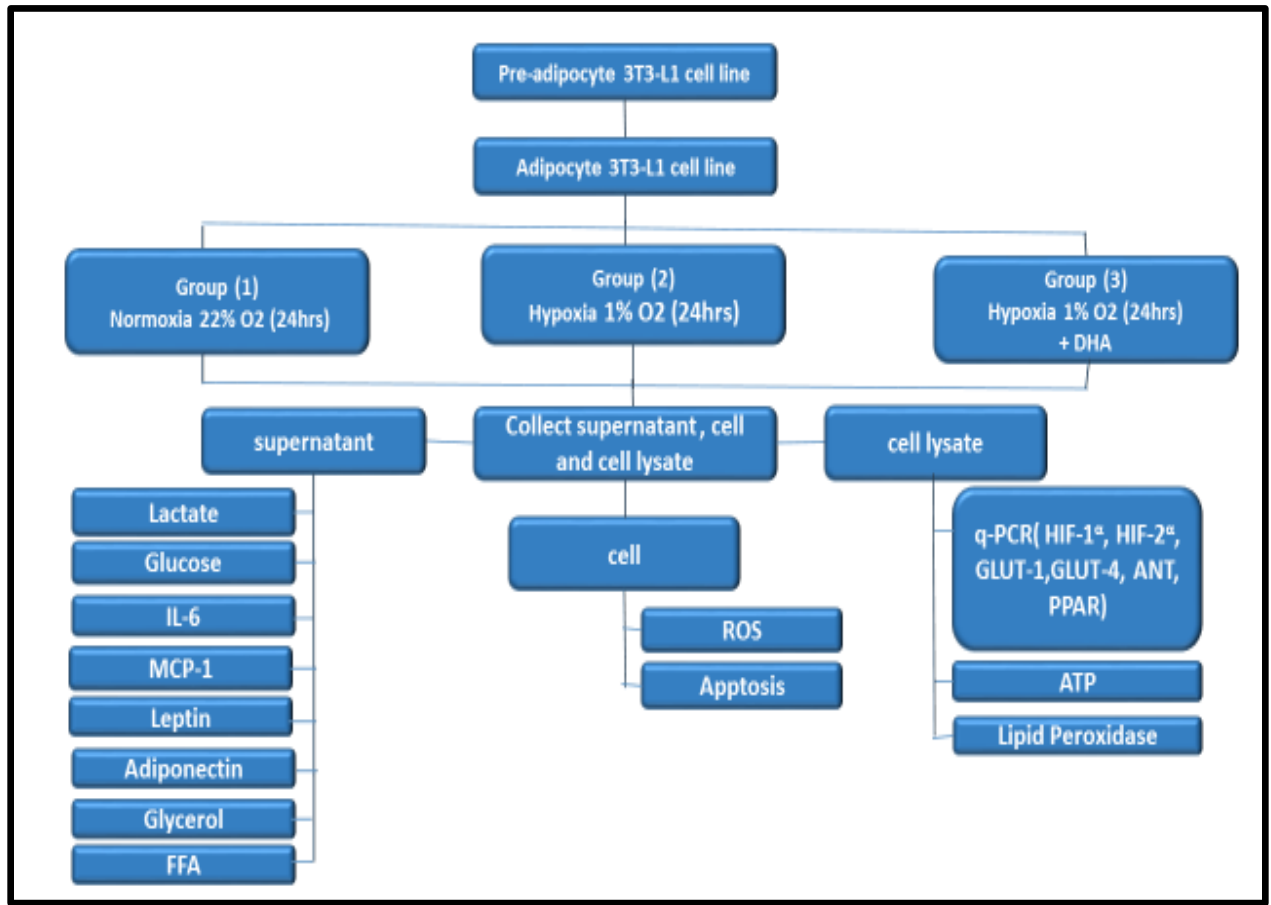
3.3. Work plan (Figure 3.1)

Figure 3.1. **Flow diagram. Sequence of testing in the work up .**This is a simplified version created with the aim of providing a general idea of the research process. First, second and third groups are shown in the following scheme. All were repeated minimum twice.

3.4. Cell Culture

3T3-L1 cell line was used in this study to model the effects of hypoxia on adipocytes cells. Cryopreserved 3T3L-1 pre-adipocytes cell passage 14 obtained from zenbio (ZenBio, Inc. 3200 East Highway 54 Suite 100 Research Triangle Park, NC, USA) were received and stored in liquid nitrogen in D105, College of Arts and Sciences building in Qatar University campus, where all cell culture work had been conducted. 3T3-L1 pre-adipocytes were expanded to passage 17, which I used for all the experiments, then the differentiation was performed following the ZenBio 3T3-L1 Cell Care Manual, with the media is explained in Figure 3.2 The media formulations are described in Table 3.2.
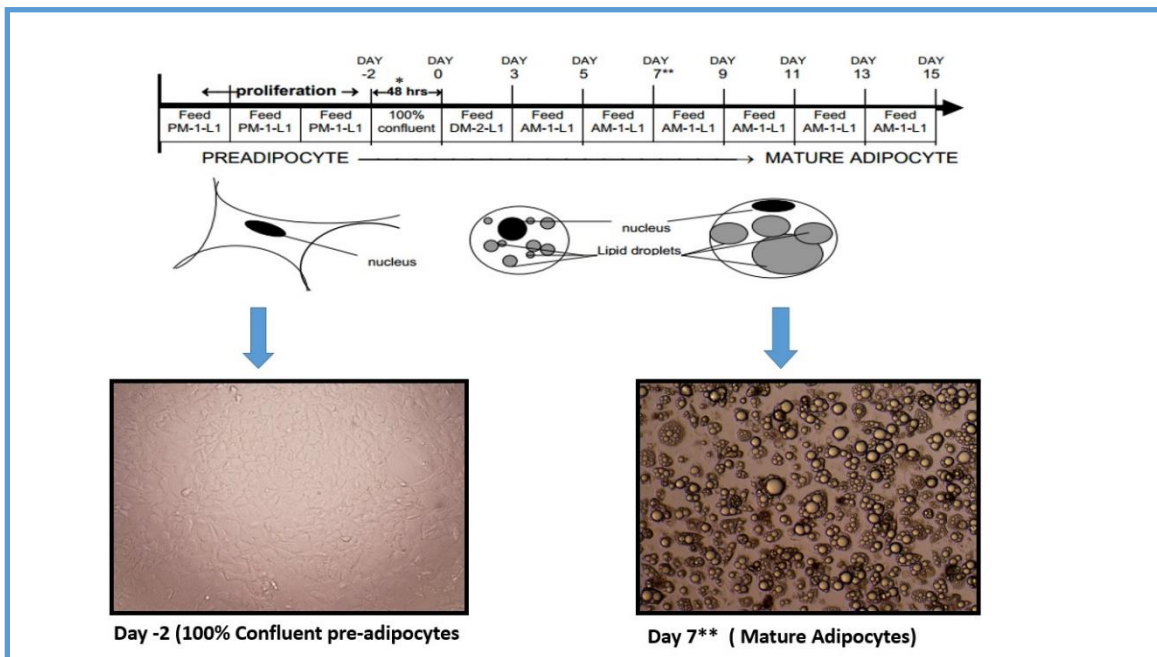

Figure 3.2. **Cell culture protocol employed.** The top Figure shows adipocyte Development by days. This figure shows the steps involved in adipocyte differentiation. After determination to the adipocyte lineage, the preadipocytes increase in number and undergo. differentiation to mature adipocyte, which can accumulate triacylglycerol and increase in size.

Table 3.2. Description of media formulations

MEDIUM	FORMULATION
3T3-L1 Preadipocyte Medium	DMEM, high glucose HEPES pH 7.4 Bovine Calf Serum (BCS) Penicillin Streptomycin Amphotericin B
3T3-L1 Adipocyte Medium	DMEM / Ham's F-12 medium (1:1, v/v) HEPES pH 7.4 Fetal Bovine Serum (FBS) Biotin Pantothenate Human insulin Dexamethasone Penicillin Streptomycin Amphotericin B
3T3-L1Differentiation Medium	DMEM / Ham's F-12 medium (1:1, v/v) HEPES pH 7.4 Fetal Bovine Serum (FBS) Biotin Pantothenate Human insulin Dexamethasone Penicillin Streptomycin Amphotericin B Isobutylmethylxanthine PPARγ agonist

3T3L-1 Cells were seeded and maintained with Pre-adipocyte Medium (PM-1-L1). The pre-adipocytes were incubated until they are 80-85% confluent (around 3-4 days), cells were provided every other day with PM-1-L1. Taking into consideration cells that never reach to 100% confluency, 3T3-L1 pre-adipocytes passage 17 were seeded in 6 well plates at a density of 50×10^3 cells per well and maintained until reaching 100% confluence, then incubated at 37°C and 5% CO_2 for an extra 48 hours. Then, the equal volume of medium was exchanged with Differentiation Medium (DM) (Day 0) and incubated for 72 h at 37°C in a humidified atmosphere containing 5% CO2. After that, the DM was exchanged with Adipocyte Maintenance Medium (AMM); AMM was changed every 48 h. Finally, the cells were fully differentiated at day 7 after induction. 3T3-L1 pre-adipocyte cells can be completely differentiated between 7 to 10 days after DM application. In case of freezing cells (Passage 16 and below), the cell pellet was resuspended in 0.5-1 ml warm cryopreserving media (amount depends on the number of cells); then 500μl of the cell suspension was placed in different 2ml cryopreserving tubes, which was stored in -80°C freezer.

3.5. Oil Red O Staining

Oil red O is fat-soluble dye, which can make fat more visible when staining. Adipocyte differentiation needed about 7 to 10 days and differentiation could be checked and confirmed by *Oil Red O Staining*. At day 10 of differentiation, 3T3-L1 adipocytes were washed with PBS, followed by fixation with 10% formalin for 6 minutes. Thereafter, washing was done using distilled water twice, and then the cells were stained for 15 minutes with oil red O solution in a 37°C. Photographs for stained cells were taken with a microscope OLYMPUS Model DP72 as shown in Figure 3.3.

5x   10x
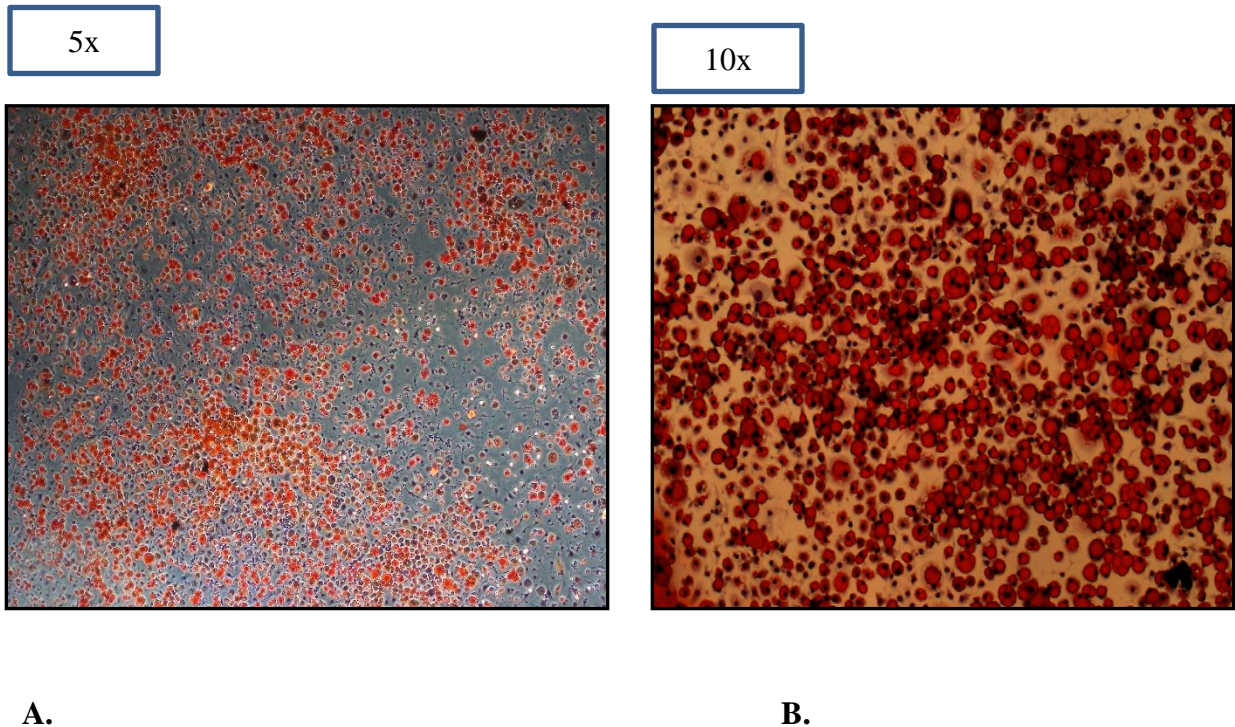
A.   B.

Figure 3.3. **Oil red O pictures of 3T3-L1 cells assessed on Day 7.** Oil red O Dye allows for visual confirmation of differentiation. Photographs for stained cells were taken with a microscope OLYMPUS Model DP72 A: magnification 5X B: magnification 10X.

3.6. Hypoxia Treatment

In place of hypoxic incubations, medium was exchanged with Basal Medium DMEM serum free medium, and adipocytes under normoxic conditions groups were incubated in 5% C O_2 and 95% air. For hypoxic conditions groups, the culture plates were placed in a Xvivo hypoxia chamber system obtain from an (Biospherix, Ca) where gas mixture was flushed consisting of 1% O_2, 94% N_2 and 5% CO_2. The third group is 3T3-L1 adipocytes were cultured for 24 hours in the presence of 50 μM DHA complexed to albumin under hypoxia 1% O_2 condition. All conditions groups were incubated at 37°C for 24 hours.

3.7. DHA treatment:

DHA (Sigma) was delivered to the cells as fatty acid/bovine serum albumin (BSA) complexes. The molar ratio of fatty acid to BSA was 4:1. DHA stock was aliquoted and stored at -20 ^{0}C.

3.8. Study groups:

Group (1): 3T3-L1 adipocytes cell with Normoxia 22% O_2 (24 hrs) treatment.

Group (2): 3T3-L1 adipocytes cell with hypoxia 1% O_2 (24 hrs) treatment.

Group (3): 3T3-L1 adipocytes cell with hypoxia 1% O_2 + DHA (24 hrs) treatment.

After hypoxia treatment, the following assays were performed:

3.9. Apoptosis Assay (Annexin V Alexa Fluor® 488):

After hypoxia, hypoxia + DHA and normoxia treatments for 24 hours, the 3T3-L1 adipocytes cells were dissociated with trypsin EDTA (Trypsinization) using 3ml TE harvested the treated cells; incubation at 37˚C for 5-7 minutes followed by centrifugation at 500x g and supernatant was discarded. The cells were resuspended in 100 µl of 1X Annexin binding buffer (ABB) and 5 µl of Annexin V Alexa Fluor® 488 in a microcentrifuge tube. The mixture was vortexed briefly and incubated in dark at room temperature for 20 minutes. This was followed by centrifugation at 500x g and resuspension in 100 µl of ABB and 1 µL of Tali® PI, brief mixing and incubation in dark at room temperature for 1-5 minutes. 25 µl of the stained cells were loaded into Tali® Cellular Analysis slide and percentage apoptosis of all samples were analyzed using Tali® Image-Based Cytometer at 488/499 nm and 535/617 nm Ex/Em wavelength for Annexin V Alexa Fluor® 488 and PI respectively. Kit use for analysis of apoptosis and dead cell count is Tali® Apoptosis Kit - Annexin V Alexa Fluor® 488 and Propidium Iodide (Life Technologies USA). The flow cytometric test was done using the Tali® Image Cytometer (Life Technologies USA).

3.10. Measurement of Reactive Oxygen Species

Percentage oxidative stress was determined by cell-rox (invitrogen) using Tali image-based cytometer. All treated cells were harvested using 3ml TE then they were centrifuged and resuspended in 200 μl PBS. 5μM (0.4 μL) CellROX® orange reagent was added, and the mixture was briefly vortexed followed by incubation at 37℃ for 30 minutes. This was superseded by centrifugation at 500x g, and the adipocytes maintenance media   removal and cells were washed three times with PBS. 25 μl of the stained cells were loaded into Tali Cellular Analysis slide and the cell viability of all samples were analyzed using Tali® Image-Based Cytometer at 545/565 nm Ex/Em wavelength. The total ROS production was qualitatively determined by the immunoflourescence of H2DCFDA (6-carboxy-2',7' dichlorodihydrofluoresceindiacetate) . After hypoxia and normoxia, and DHA treatments for 24 h, the 3T3-L1 adipocytes were incubated in 2ml of pre-warmed PBS and 10μM (2μl) Carboxy-H2DCFDA dye for 30inutes at 37˚C. The buffer was then replaced with pre-warmed growth medium and multiple fluorescent images were captured using immunofluorescent inverted microscope Olympus Microscope BX51 Fluorescence.

3.11. Lipid peroxidation (MDA) Assay

By using Lipid peroxidation malondialdehyde (MDA) assay kit (Abcam, USA) the amount of lipid peroxidation was measured which estimated the oxidative stress in the cells. After hypoxia, normoxia and DHA treatment, the condition media was collected and removed and the cells were washed twice by cold PBS. The cells were homogenized at a density of 5×10^5 cells per sample

in the MDA Lysis Buffer in ice. After efficient lysis was confirmed after checked under microscope for homogenesis, the cell lysate was centrifuged and supernatant was collected. TBA (Thiobarbituric Acid) reagent was added to the sample and the standard and incubated at 95 ^{0}C. The samples were cooled for 10 min at room temperature then measurement made in OD 532 nm by the microplate reader Tecan. Data are expressed as nM MDA released.

3.12. <u>Lactate & Glucose uptake measurements</u>

Using cobas b 123 POC, glucose and lactate were measured in the MSS (Metabolite Sensitive Sensors) measuring chamber, by the MSS cartridge, which is a multi-parameter sensor. Glucose is oxidized by glucose oxidase and atmospheric oxygen to form gluconolactone. Lactate is oxidized by lactate oxidase to form pyruvate. The H_2O_2 generated from these reactions is determined amperometrically using manganese dioxide/carbon electrodes at 350mV. Calibration of the MSS parameters takes place using 4 solutions, whose weighing concentration forms the basis for determining the measured values. All MSS calibrations are referenced to a reference measurement using a standby solution. This reference measurement is carried out after the calibration solution has been measured.

The glucose uptake was measured using the below equation as previously published (Hashimoto et al., 2013):

Glucose uptake = (A – B) / C

A= Glucose conc. in CM (Condition Medium) pre-treatment

B= Glucose conc. in CM post-treatment

C= Treatment duration (per hours)

3.13. Glycerol and Free Fatty Acid release measurement

All *in vitro* lipolysis experiments were performed with 7 days post-differentiated 3T3-L1 adipocytes, grown and differentiated in 96- well tissue culture plates. 3T3-L1 Adipocytes were equilibrated (0.5–1 h at 37 °C) in fresh DMEM/10 % (v/v) FBS (Fetal Bovine Serum

), and then were washed twice with PBS (pre-warmed to 37 °C). To initiate lipolysis, PBS was removed, and replaced with 0.5 ml of KRH buffer [25 mM HEPES (pH 7.4), 125 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 2.5 mM $CaCl_2$ and 2.5 mM $MgCl_2$], containing 3 % fatty-acid-free BSA (Intergen) and 5 mM glucose. Treatment with hypoxia, DHA and normoxia was carried out as indicated at 37 °C in a 5 % CO_2 atmosphere for periods 24 h, after which the medium was collected for glycerol & FFA analyses, Glycerol & FFA content, were determined using a lipolysis assay kit (ZenBio, USA) according to the manufacturer's instructions.

The optical density of each test is then measured at 540 nm by the microplate reader Tecan. Data are expressed as µM glycerol or FFA released.

3.14. Estimation of Inflammatory Cytokines, Chemokine's and Hormones –Elisa

IL-6, MCP-1, Leptin and Adiponectin were estimated in the conditioned media. Samples were run in duplicate using enzyme-linked immunosorbent assay (ELISA) kits (BioVendorInc.Int). No specific modifications were done; the steps were done based upon the manufacturer's protocol.

3.15. ATP measurements

Cell lysate all treated cell ATP measurements were determined using the ATPColorimetric/Fluorometric Assay kit (Abcam). Measurements were performed with the fluorometric assay using VICTOR 3V Multilabel Plate Readers, Perkin Elmer, Massachusetts, USA. Following the manufacturer's instructions, on 1×10^6 3T3-L1 cells. ATP contents was measured in duplicate and calculated per mM.

3.16. Quantitative real-time RT-PCR

The total RNA was extracted using the TRIzol protocol (Life Technologies, USA) from treated 3T3L-L1 adipocytes. Mouse Actb primer (Mm00607939_s1) was used as an endogenous control for normalization of mRNA. The cDNA synthesis from the isolated RNA was performed using a High Capacity RNA-to-cDNA kit (Applied Biosystems, USA). The primer sets are shown in Table. (1) Quantitative real-time PCR was performed in a reaction containing cDNA and TaqMan Gene Expression Master Mix (Applied Biosystems, USA). Samples were analyzed with the Applied Biosystems 7500 Real-Time PCR System (Applied BioSystems, USA). All PCRs were done in triplicate. The next paragraphs explained such steps in details:

3.17. Total RNA Extraction

Total RNA was extracted from the cells that were preserved in TRIzol reagent at -80° C, according to the protocol provided by the manufacturer (REF #15596026, Ambion RNA by Life

Technologies, USA). The tubes containing the cell extract in 1ml TRIzol each were taken out of freezer and left to be thawed. Then, 200µl chloroform was added

to each tube; then the tubes were shaken and incubated in room temperature for 2-3 minutes. After that, the tubes were centrifuged for 15 minutes at 13000 rpm and $4^{o}C$, whereas the mixture separated into a lower phenol-chloroform phase, an interphase, and a colorless upper aqueous phase . The colorless upper phase contained RNA, which was harvested and placed in a clean-labeled microcentrifuge tubes. 500µl of 100% isopropanol was added on each tube and incubated in $-20^{o}C$ for 1 hour or overnight. After that, the tubes were centrifuged for 10 minutes at 13000 rpm and $4^{o}C$. RNA formed a pellet on the bottom and side of the tubes. The supernatant was discarded without disturbing the pellet. 1ml 70% Ethanol was then added on each tube for washing and mixed slightly. Again, the tubes were centrifuged for 5 minutes at 13000 rpm at $4^{o}C$. The supernatant was removed completely without disturbing the pellet, and then left to air dry. RNA pellet was then resuspended in 30µl RNase- free water and mixed well then placed in heat block at $55^{o}C$ for 10 minutes. After that, the tubes were placed on ice and RNA concentration was measured using NanoDrop.

3.18. cDNA Synthesis

cDNA was prepared according to the manufacturer protocol of High Capacity RNA-to-cDNA kit supplied by the company (P/N 4387406, Applied Biosystems, USA). 2 tubes from each day of the three experiments (of the three groups) were selected according to RNA quality and concentration. The reaction mix was prepared then distributed on all labeled PCR tubes. After that, RNA and water were added with different quantities each according to RNA concentration.

Generally, the cDNA synthesis mixture had the following components, as shown in the following Table:

Table 3.3. cDNA synthesis mixture

Component	Volume/Reaction (µl)
2X RT Buffer	10.0
20X Enzyme Mix	1.0
RNA Sample	1-9 (according to concentration)
Nuclease-free H_2O	1-8 (according to RNA sample volume)
Total per Reaction	20

The tubes were caped and mixed by vortex then spun down before placing them in thermo-cycler (please add the name of PCR, model, company). Reverse transcription reaction was performed according to manufacturer's protocol: $37^{o}C$ for 60 minutes followed by $95^{o}C$ for 5 minutes then hold at $4^{o}C$. cDNA tubes were kept in the freezer at $-20^{o}C$.

3.19. Real Time PCR

For gene expression experiments, Applied Biosystems real-time quantitative PCR7500 instrument was used. TaqMan Gene Expression Master Mix (P/N 4369016) from Applied Biosystems was used as well. the PCR reaction mix was prepared as in Table (3.4)

Table 3.4. qPCR mixture

Content	Amount per sample
Master Mix	10 µl
Primer	1 µl
RNA free H_2O	8 µl
cDNA (200ng)	1 µl
Total	**20 µl**

19 µl from each mixture were distributed in the corresponding wells in the optical 96-well plate, and then 1 µl from each cDNA sample was added. The plate was then sealed with adhesive cover and centrifuged, and then was placed in the real-time PCR instrument.

Another mixture was performed exactly the same for β-actin as endogenous control. Cycle threshold (CT) defined as the cycle number at which a significant increase in the fluorescence signal compared to background emission is first detected. Calculating CT values for each sample was performed to quantify unknown samples. ΔCT defined for each sample by subtracting the β-actin gene CT from each selected gene CT. Relative gene expression was calculated by the 2-ΔΔCT methods, i.e. as the negative exponential power of 2 of each sample's ΔCT minus the ΔCT of a reference sample, which kept constant throughout experiments. Relative gene expression was calculated automatically by Sequence Detection Systems Software version 2.3.

3.20. Statistical Data Analysis

Each experiment was conducted at least three times (3biological replicates/ 3 times). A mean value and SD) of three independent experiments is presented for all assays and data normality is checked using Shapiro-Wilk test. Comparisons between groups were done using one-way ANOVA analyses and the Bonferroni post-hoc test plus Student's t-test with the Prism 6 (GraphPad Software, San Diego CA USA) graphics/statistics package. Two-tailed p value is significance at $P \leq 0.05$.

Chapter 4

4. Results:

4.1 Effect of hypoxia [1% O_2] compare to DHA treated group on apoptosis and necrosis in 3T3-L1 cells

To check apoptosis and necrosis for **3T3-L1** cells, Tali® Image-Based Cytometer was used after the 3T3L-1 cells from the all study groups were stained with green Annexin V – Alexa Fluor® 488, which stains necrotic cells with both red Propidium iodide and green Annexin V – Alexa Fluor® 488, and does not stain live cells. The results presented here showed that apoptosis and necrosis of the adipocytes were significantly increased in cells treated with hypoxia by 13% and 20%, respectively with (P<0.05) compare with normoxia. On the other hand, DHA treatment significantly reduces the apoptosis by 29% and the necrosis by 27% in the hypoxic cells with (P<0.05) as shown in (Figure 4.1).

The results of apoptosis and necrosis for cell, which treated with DHA and hypoxia, show significant decrease compared with hypoxia treatment with (P<0.05) as shown in (Figure 4.1).
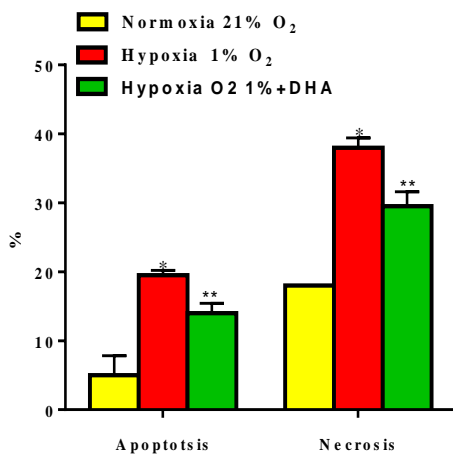

Figure 4.1. Assessment of the number of apoptotic and necrotic cells under different levels of oxygenation. *Data* represent the percentage of apoptotic and necrotic cells under normoxia [control group] , hypoxic conditions and hypoxia +DHA (50uM) condition . 3T3-L1 adipocytes (7 days post-differentiation) were incubated for 24 hours continuously in 21% O_2 and 1% O_2, respectively. *P≤ 0.05 compared with the normoxic group [control]. **P≤0.05 compared with hypoxia 1% O_2 group.

4.2. Effect of hypoxia [1% O_2] compare to DHA treated group on oxidative stress (ROS) in 3T3L-1 adipocytes

Oxidative stress measured by the products of the (reactive oxygen species; ROS) in percentage (%) was determined quantitatively by the cell-rox dye (Invitrogen) using Tali image-based cytometer. The total ROS production increased significantly in 3T3L-1 (7 days post-differentiation) treated with hypoxia [1% O2] by ≈ 2 folds compared to cells exposed to normoxia [21% O_2] with (P≤0.05). DHA treatment significantly reduces the ROS by 24% in the hypoxic cells with (P=0.0164) as shown in Figure 4.2.

In addition, the qualitative determination of the total intracellular ROS by staining of the treated and untreated cells with H2DCFDA dye using the immunofluorescence microscope showed marked increase in ROS spots in 3T3L-1 adipocytes exposed to hypoxia compared with the other two group as shown in Figure 4.2.
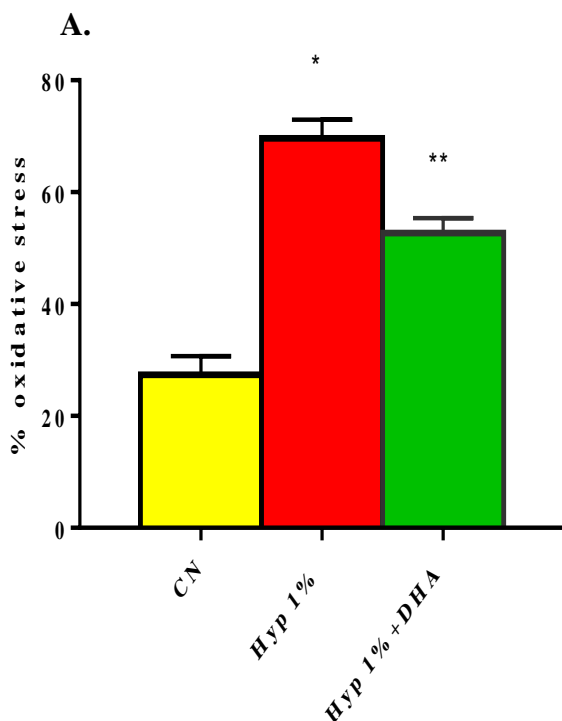
Figure *4.2.*. Oxidative stress levels in response to variations in oxygenation and the impact of DHA. A. Data set represent the percentage of % oxidative stress percentage of cells under normoxic [control group], hypoxic conditions treated for 24 hours and hypoxic conditions treated for 24 hours plus 50uM of DHA in 3T3-L1 adipocytes (7 days post-differentiation). *P<0.05 is significant compared with the normoxic [control]. **P≤0.05 compared with hypoxia 1% O_2 group.

B.
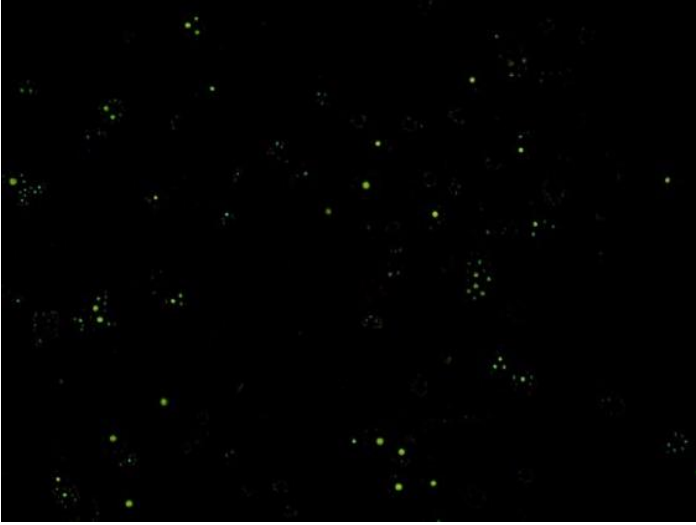
CN
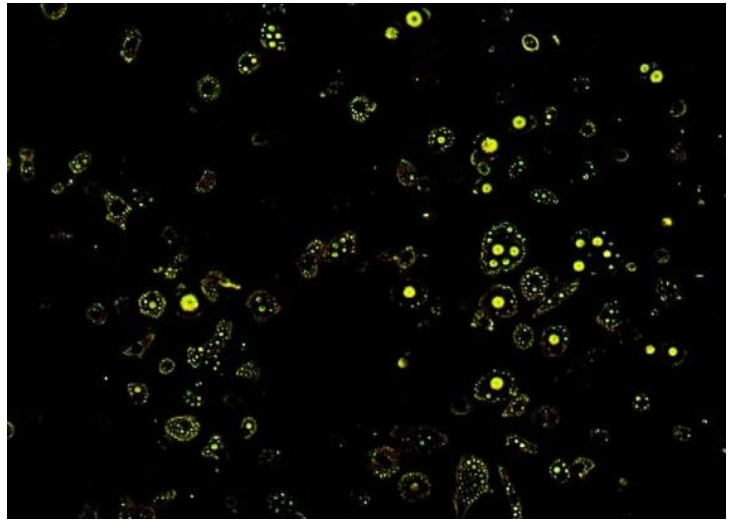
Hypoxia 1%

Hypoxia 1% +DHA

B. Total ROS production increased in 3T3L-1 treated with hypoxia compare with both group normoxia and DHA treated group as per qualitative determination of immunofluorescence of H2DCFDA stained cells. X5

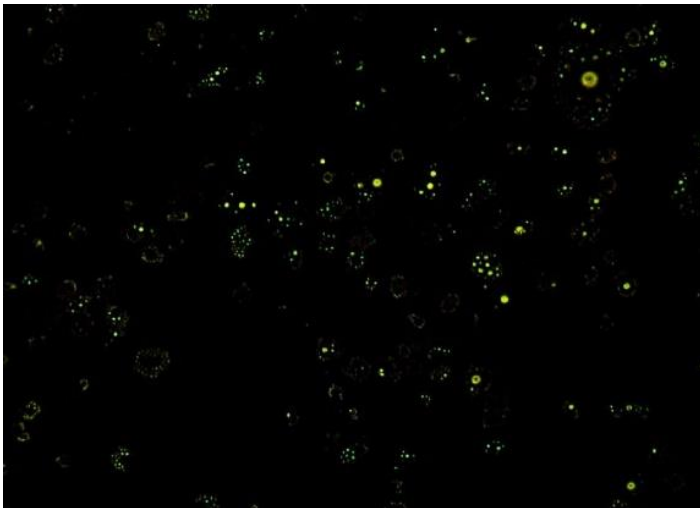**4.3 Impact of hypoxia 1% O$_2$ and DHA on lipid peroxidation; malondialdehyde (MAD)**

The lipid peroxidation was determined in the cell lysate of both groups. The level [mean ±SD] of lipid oxidative stress marker MDA was significantly higher in the hypoxic group (1.89± 0.053 nM) than the controls (1.61±0.036nM), p value=0.0119 as shown in Figure 4.3. DHA treatment insignificantly reduces the MAD by 4% in the hypoxic cells with (P=0.224) as shown in Figure 4.3.
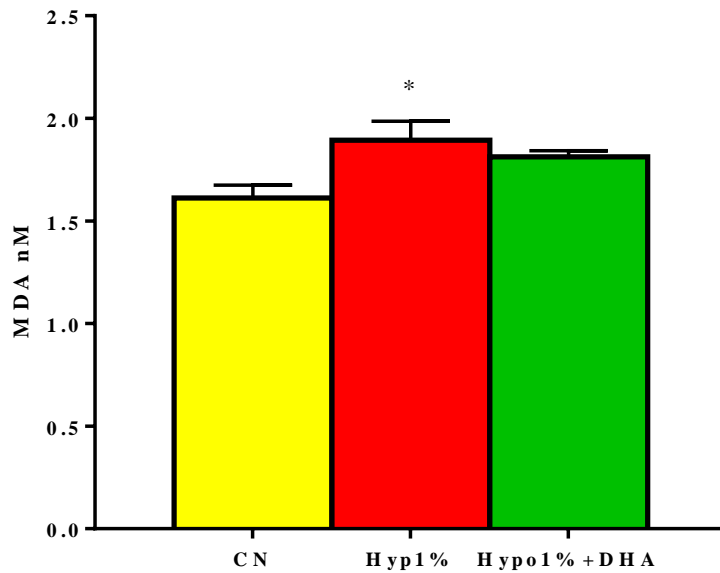
Figure 4.3. Lipid peroxidation in response to levels of oxygenation and the impact of DHA.
The results are given as the means ± S.D of 3 independent experiments in duplicate. * p≤0.05 is statistically significant vs. controls.

4.4. Effect of Hypoxia 1%O_2 and DHA on lipid metabolism

Lipolysis was evaluated with glycerol and free fatty acid release into the culture medium of 3T3-L1 adipocytes after exposure to hypoxia and normoxia for 24 h as detailed in the methods section. As shown in Figure 4.4, the glycerol and the free fatty acids release is significantly increased in the 3T3-L1 adipocytes treated with hypoxia 1% O_2 by ≈1.5 folds, and ≈2 folds compared with the normoxic cells, with p values are 0.0004 and 0.0066, respectively. The glycerol and the free fatty acid release is significantly decreased in the hypoxic cells treated with, DHA by 18%, and 55% compare with hypoxia treatment, P values =0.050 and 0.0146, respectively.

A.

B.
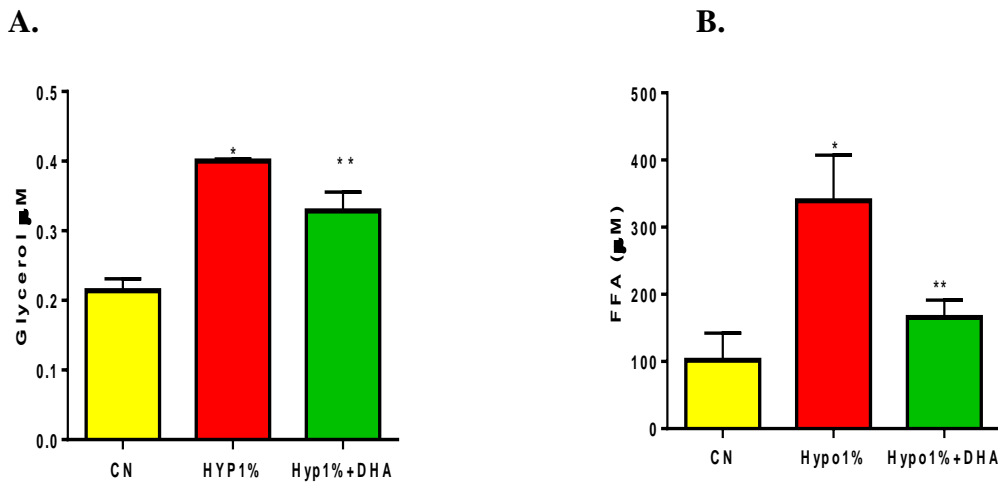
Figure 4.4. Glycerol and fatty acid release in response to levels of oxygenation and the impact of DHA. A. Glycerol and B. FFA release (μM) into the culture medium of 3T3-L1 adipocytes [7 days postdifferentitaion] after exposure to hypoxia [1% O_2] hypoxia +DHA and normoxia exposure for 24 h. Data set represent the mean ±SD of 3 independent experiments *p<0.05 indicates the significant difference between hypoxia vs. normoxia treated cells. **P≤0.05 compared with hypoxia 1% O_2 group.

4.5. Glucose Uptake and Lactate level after expose to hypoxia and DHA treatment

As shown in Figure 11, the mean ±SD of the glucose uptake was significantly higher in the hypoxic group (0.380± 0.065 mmol/24h) than the control group (0.223±0.012 mmol/24h), and *p* value=0.0013. DHA treatment significantly reduces the glucose uptake (0.270± 0.035 mmol/24h) by 29% in the hypoxic cells with (P=0.0454) as shown in Figure 4.5.
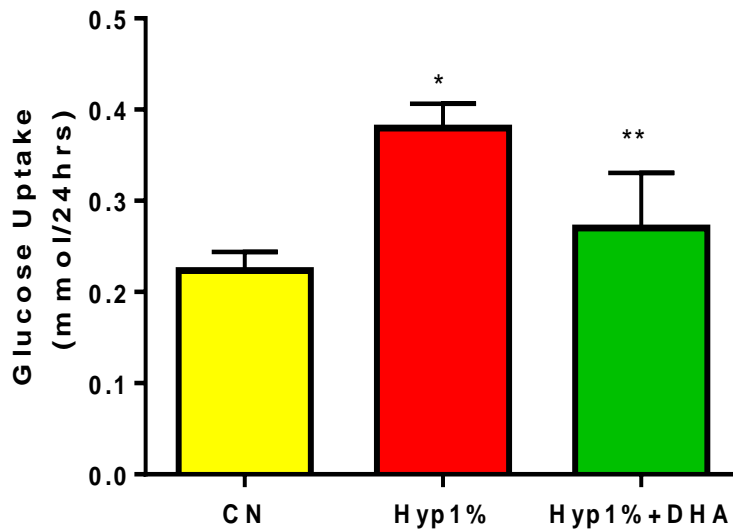
Figure 4.5. Glucose uptake in response to levels of oxygenation and the impact of DHA. Glucose Uptake (mmol/24h) in 3T3-L1 adipocytes [7 days post-differentiation] after 24hrs of normoxia, hypoxia, and hypoxia +DHA. The results are given as the means ± S.D for 3 independent experiments. * p≤0.05 is statically significant than the control (normoxia). **P≤0.05 compared with hypoxia 1% O_2 group.

Lactate release is significantly increased in the 3T3-L1 adipocytes (7-10 days post-differentiation) treated with hypoxia 1% O_2 for 24hrs, by ≈2 folds compared with untreated cells (normoxic), with p values = 0.0007. DHA treatment significantly reduces the lactate release (9.5mmol) by 40% in the hypoxic cells with (P=0.009).
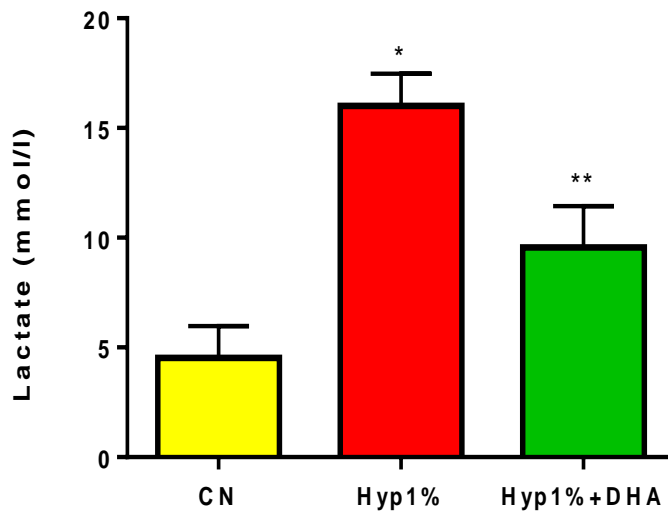
Figure 4.6. Lactate production in response to levels of oxygenation and the impact of DHA. b. Lactate release in 3T3-L1 adipocytes CM [12 days post-differentiation] after 24hrs of normoxia, hypoxia, and hypoxia +DHA treatment. The results are given as the means ± S.D for 3 independent experiments. * p≤0.05 is statically significant than the control (normoxia). **P≤0.05 compared with hypoxia 1% O_2 group.

4.6. pH level in the condition medium after hypoxia and DHA treatment:
pH level measured in both media after 24 hour of normoxia and hypoxia treatment showed a significant decrease in hypoxia pH medium (7.034±0.021) compared to Normoxia pH medium (7.410± 0.025), p=0.035 . DHA treatment significantly increased the pH (7.238 ± 0.04901,n=3) compared with the hypoxic group, (P=0.0189).
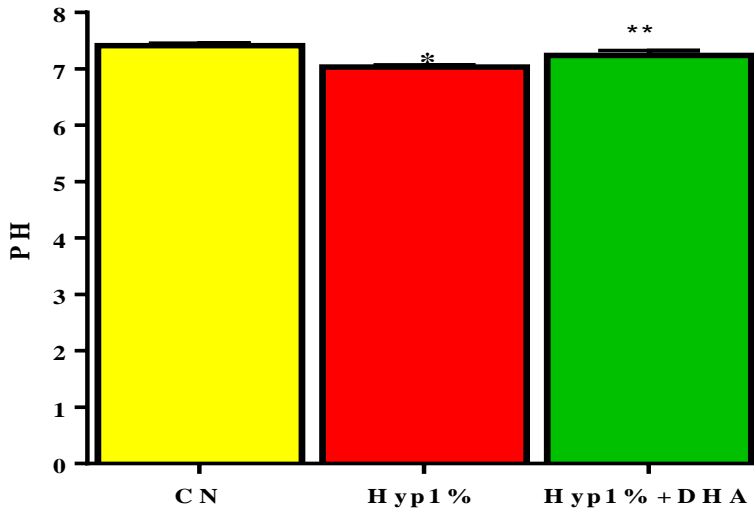
Figure 4.7. pH changes in response to levels of oxygenation and the impact of DHA. pH level in 3T3-L1 adipocytes CM [7 days post-differentiation] after 24hrs of normoxia, hypoxia, and hypoxia +DHA. The results are given as the means ± S.D for 3 independent experiments. * p≤0.05 is statically significant than the control (normoxia). **P≤0.05 compared with hypoxia 1% O_2 group.

4.7 ATP level in cell after DHA and Hypoxia treatment

ATP was quantification from cell lysate in both groups after exposure to hypoxia and normoxia condition as described previously in methodology. As shown in Figure 14 the level [mean ±SD] of ATP in mM was significantly lower in the hypoxic group (6.97± 0.367) than the controls (21.76 ±1.086), with p value =0.007. While DHA treatment showed no significant effect on APT content (6.55±0.16) compared to hypoxic cells,   p= 0.353.
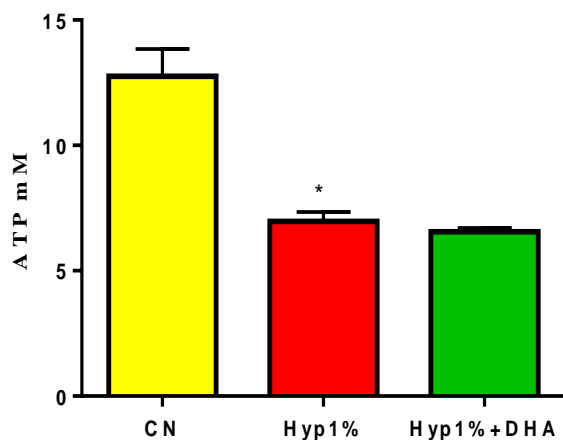
Figure 4.8. ATP level in response to levels of oxygenation and the impact of DHA. 3T3-L1 adipocytes [7 days post-differentiation] after 24hrs of normoxia, hypoxia, and hypoxia +DHA treatment. The results are given as the means ± S.D for 3 independent experiments. * p≤0.05 is statically significant than the control (normoxia).Significantly different: * p≤0.05, vs. controls. **P≤0.05 compared with hypoxia 1% O_2 group.

4.8. The effects of hypoxia on adipokine release

I examined the release of distinct adipokines from 3T3-L1 adipocytes after 24 hour exposure to hypoxia and normoxia. The results expressed showed that hypoxia 1% O_2 significantly increases the secretion of the followings; Il-6 (775.80 $\pm$ 36.45 pg/ml) compared to the normoxic cells (56.76 $\pm$ 8.926 pg/ml), p =<0.0001; MCP-1 (83.07 $\pm$ 1.696 pg/ml) compared to the normoxic cells (476.20 $\pm$ 36.25 pg/ml), p =0.0004 and; leptin (2.571 $\pm$ 0.053 ng/ml) compared to the normoxic cells (1.741 $\pm$ 0.01530 ng/ml), p =0.0001, respectively. On the contrary, hypoxia significantly decreases the release of adiponectin from3T3-L1 adipocytes (3.667 $\pm$ 0.264 µg/ml) compared to the normoxic cells (18.690 $\pm$ 0.096 µg/ml), p <0.0001, respectively.

DHA treatment to hypoxic cells significantly decreased the secretion of; Il-6 (554.5 $\pm$ 44.4 pg/ml) compared to hypoxia 1% O_2 (775.80 $\pm$ 36.45 pg/ml), P =0.0183 and; leptin (2.27 $\pm$ 0.038 ng/ml) compared to hypoxia 1% O_2 (2.571 $\pm$ 0.053 ng/ml), P =0.0099, respectively. On the contrast, hypoxia 1% O_2 +DHA significantly increased the release of adiponectin from 3T3-L1 adipocytes (9.584$\pm$ 0.12 µg/ml) compared to the hypoxic cells (3.667 $\pm$ 0.264 µg/ml), p <0.0001, respectively, as shown in Figure 15.

DHA significantly reduced the MCP-1 in hypoxic cells, p= (0.0476)

A.
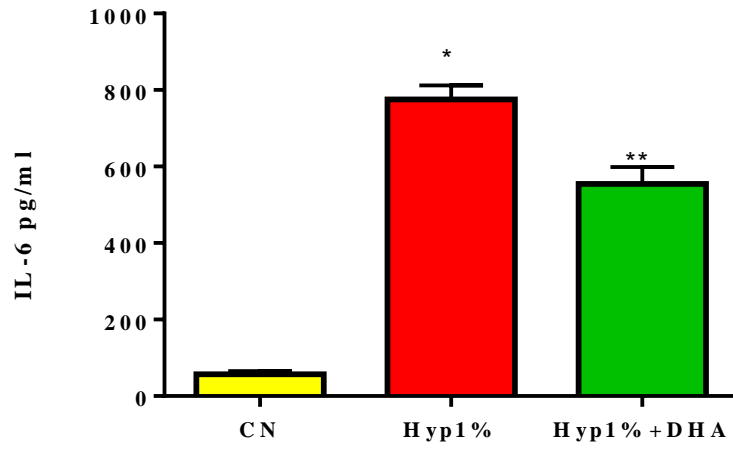
B.
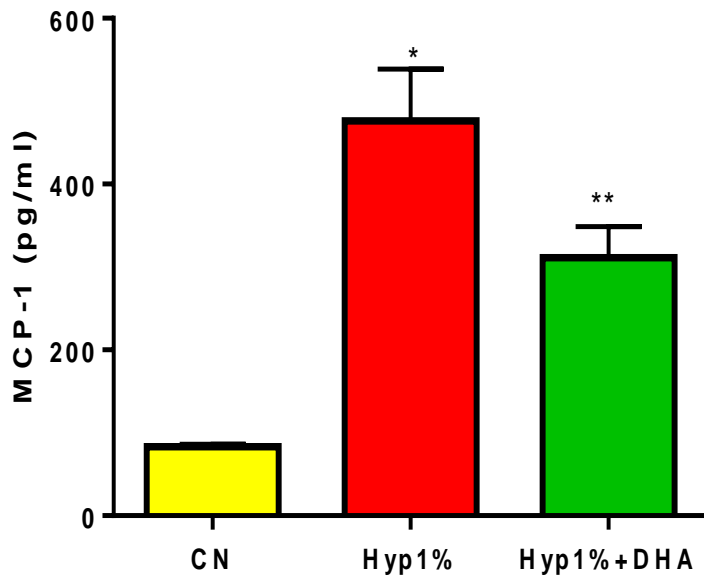
C.
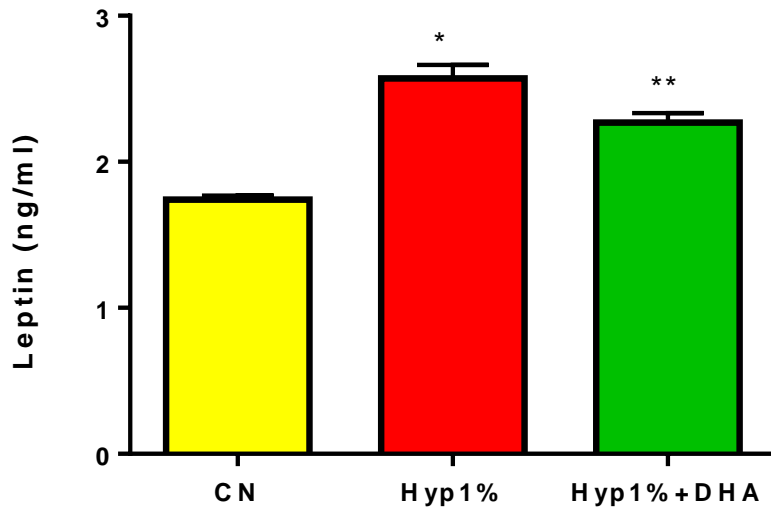
D.
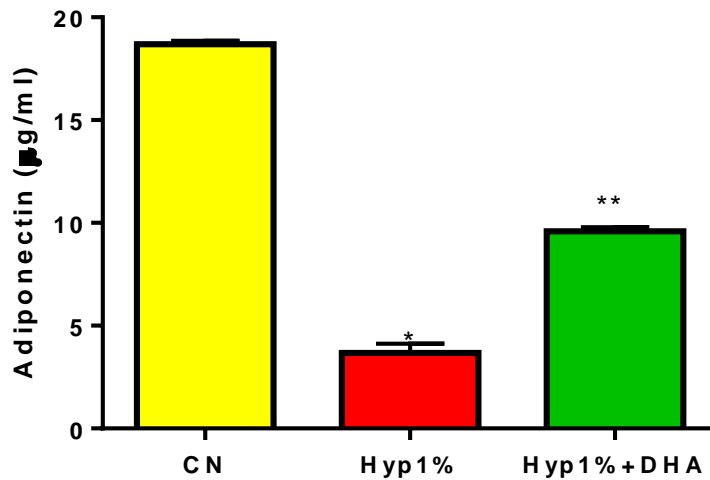
Figure 4.9. Estimation of adipokine release by ELISA in response to levels of oxygenation and the impact of DHA. (A) Interleukin 6 (IL- 6), (B) MCP-1, (C) Leptin, (D) Adiponectin. Values are means with SD represented by vertical bars for 3 independent experiments. * Mean value is significantly different from the normoxic (control, CN) cells ($P<0.05$). **$P\leq0.05$ compared with hypoxia 1% O_2 group.

4.9. Hypoxia & Gene expression

Expression of hypoxia-responsive genes was tested by qPCR to define the hypoxia response. The genes include HIF1α, HIF2α, GLUT1, GLUT4, PPARγ, and ANT2.

HIF1α, HIF2α expression increased significantly in 3T3L-1 treated with hypoxia by ≈ 3 folds and ≈ 4 folds compared to cells exposed to normoxic [21% O_2] with p values are 0.0019 and 0.0003, respectively. GLUT1, GLUT4 increased significantly in 3T3L-1 treated with hypoxia by ≈ 15 folds and 1.7 folds compared to cells exposed to normoxic [21% O_2] with p values are 0.006 and 0.023, respectively. PPARγ, ANT2 decrease significantly in 3T3L-1 treated with hypoxia by 87% and 79 % compared to cells exposed to normoxic [21% O_2] with p values of 0.0001 and 0.008, respectively.

While HIF1α, HIF2α and GLUT1 expression degreased significantly in cell treated with DHA plus 1% **O_2** hypoxia by 40%, 32% and 65%, respectively compare with cell treated with only 1% **O_2** hypoxia (Figure 4.9).

A.
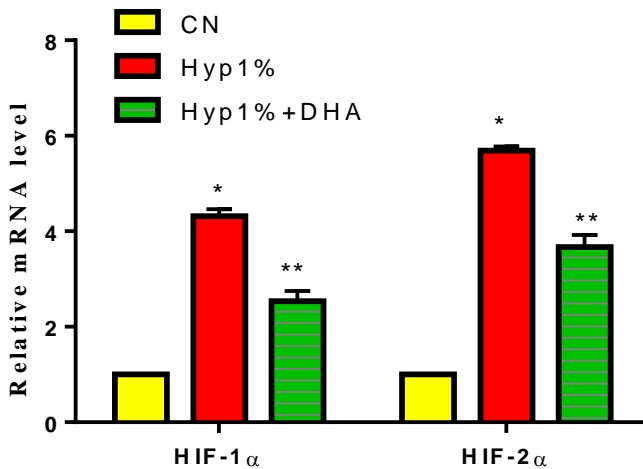
B.
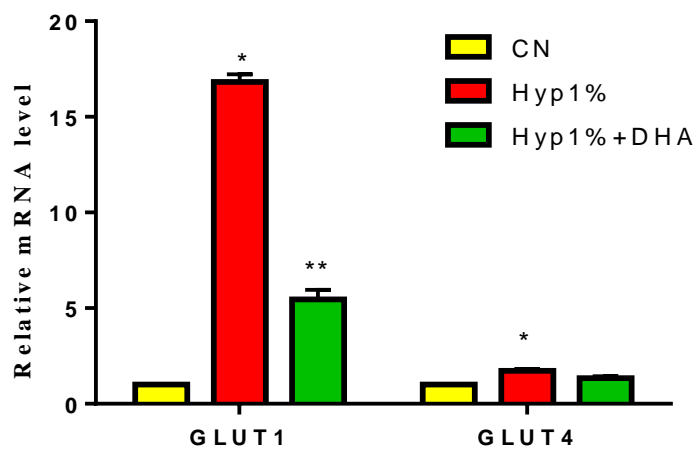
C.
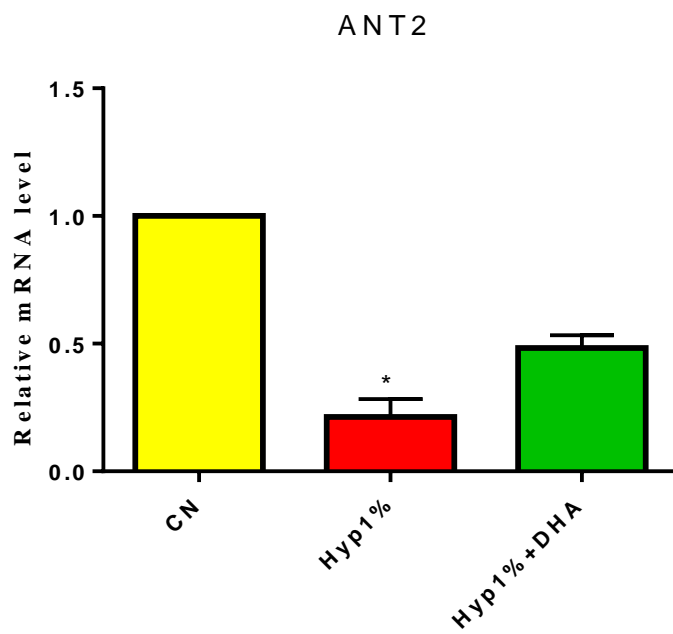
D.
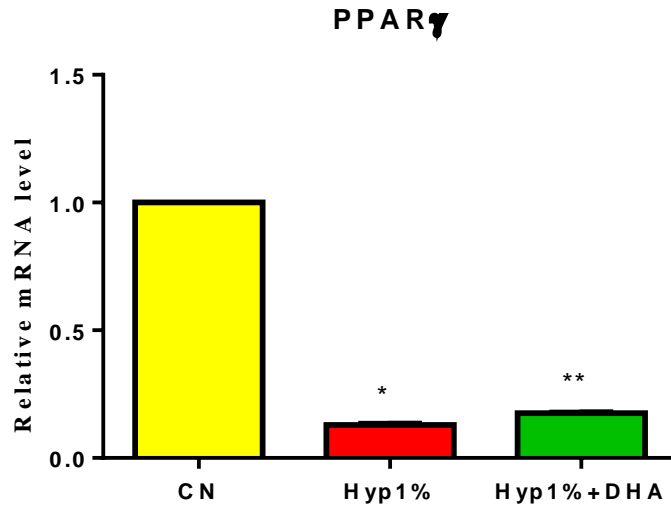
Figure 4.10. Hypoxia-induced gene expression in response to levels of oxygenation and the impact of DHA. Relative mRNA levels were determined by quantitative RT-PCR for 3 independent experiments. * Panel A; HIF1, HIF2, panel B:GLUT1 and GLUT4, pANELc: ant2 AND Pnael D: PPARy. Mean values with SD is significantly different from the control cells (P<0.05). **P≤0.05 compared with hypoxia 1% O_2 group.

Chapter 5

5.1 Discussion

Extreme enlargement in the size of adipose tissue is a hallmark of obesity that causes many complications such as cancer, T2DM and CVD (Kopelman, 2000). One of the leading factors in obesity that contributes to the dysfunctions of adipocytes is hypoxia. Oxygen plays an important role in the metabolic regulation of carbohydrate and lipids in many tissues (Katz & Sahlin, 1989). Oxygen tension in the human body is lower than the external environment and is variable in different tissues ranging from 0.5% to 14.0% (Duling & Pittman, 1975). In the present study, I investigated the impact of hypoxia (1% O_2) on mature adipocytes of murine origin 3T3-L1 on oxidative stress, metabolic substrates, ATP content and the adipokines released with the genes involved in hypoxia and to understand and mimic feature of in vivo adipose tissue hypoxia. Moreover, I studied if DHA, polyunsaturated fatty acids, can counteract the hypoxic effects on adipocyte functions. To our knowledge, this is the first study done to examine the effects of DHA on hypoxic adipocytes in vitro.

In the current study, 3T3-L1 murine adipocytes were incubated with (1% O_2) for 24 hrs as previously published (West, Prinz, Francendese, & Greenwood, 1987). Such level of hypoxia

(1% O_2) is equal to 7.6mm Hg of PO_2 which is close to the level detected in WAT of obese mouse (ob/ob) (Trayhurn, 2013). The present study revealed that exposure of mature adipocytes to hypoxia (1% O_2) for 24 hours significantly modified the mitochondria functions with metabolic substrates of carbohydrate and lipid metabolism. It also provokes the inflammation, increaes the reactive oxygen species, lipid peroxidation and induces apoptosis and necrosis. In addition, this hypoxia upregulates the hypoxia responsive genes; such as HIf1 and HIf2; glucose transporter gene; GLUT1 with reduction of adipogenic gene; PPARγ. DHA treatment to hypoxic cell modified such hypoxic effects by counteract the effects of hypoxia. These findings and these implications will be further discussed in the following paragraphs.

Local hypoxia occurs due to reduced oxygenation in adipocytes tissue of the obese subject. Previous study showed that exposure to hypoxia increases inflammatory adipokine and transcription of genes responsible for metabolic stress in adipose tissue (O'Rourke et al., 2011). Another factor that cause insufficient adipose tissue oxygenation is a reduction of blood flow due to large adipocytes size in the obese subject (Brook et al., 1972).

Hypoxia activated HIF1α gene and consequently modified the expression of nearly 70 genes (He et al., 2011; Trayhurn, Wang, & Wood, 2008). The genes activated by HIF1α are involved mainly in cell oxidative stress, inflammation, glucose metabolism, angiogenesis and apoptosis (Trayhurn et al., 2008). The main function of these genes is to assist cell survival by stimulating angiogenesis and glycolytic metabolism in low oxygen conditions (Bento & Pereira, 2011). Stimulation of transcript elements such as HIF1α performing a vital role in the stimulation of

inflammatory chemokine and cytokines which include leptin, adiponectin, IL-6, VEGF, PAI-1 and MCP-1 by hypoxia (Quintero, Gonzalez-Muniesa, Garcia-Diaz, & Martinez, 2012). These cytokines are likely involved in macrophage infiltration into adipose tissue (Bastard et al., 2006). In the current study, both IL-6 and MCP-1 secretion by the 3T3-L1 adipocytes is increased markedly by exposure to hypoxia. These findings are consistent with previous studies (S. Famulla, Horrighs, Cramer, Sell, & Eckel, 2012; Quintero, Gonzalez-Muniesa, & Martinez, 2012). IL-6 is considered as an inducer of an acute-phase reaction that are present in high-stress levels and the presence of an inflammatory or infectious disease. Genetically IL-6 deficient mice develop mature onset obesity that is partly reversed when administered with IL-6 (Papanicolaou et al., 1998). The role of the MCP-1/CCR2 in obesity-induced inflammation remains incompletely understood, as not all studies found the influence of MCP-1 deficiency relevant to macrophage accumulation in adipose tissue or insulin sensitivity (Kirk et al., 2008). DHA treatment reduces the secretion of the proinflammatory cytokines IL-6 and MCP-1, consistent with previous studies (Cranmer-Byng, Liddle, De Boer, Monk, & Robinson, 2015; De Boer, Monk, & Robinson, 2014).

One of the most interesting results of the present study is the marked reduction in release and secretion of adiponectin in mature adipocytes exposed to hypoxia 1% O_2, which is consistent with previous studies (S. Famulla et al., 2012; Guerre-Millo, 2006). The decreased secretion of adiponectin hormone is involved in the insulin resistance, metabolic syndrome, inflammation and the angiogenesis (Cummins & Taylor, 2005; Nigro et al., 2014). Adiponectin secretion is dependent on O_2 level as shown in previous studies (Susanne Famulla, Schlich, Sell, & Eckel, 2012) and in unpublished data from our lab, whereas 10% O_2 increased the adiponectin secretion

by 2.2 folds over 1% O_2 while 21% O_2 increased by 3.6 folds over the secretion in 1% O_2. Moreover, the current study showed increased leptin secretion in adipocytes exposed to hypoxia. These data are supported by previous studies (Bekhite et al., 2014; Macrea, Martin, & Horowitz, 2011). Leptin is a hormone which regulates energy balance and body weight, (Weir, Robertson, Leigh, Vass, & Panteleyev, 2011) and hyperleptinemai is involved in insulin resistance, which may explain why hypoxia could induce insulin resistance by increased secretion of pro-inflammatory cytokines such as Il-6, MCP-I and leptin with decreased adiponectin as shown in the current study and previous studies (Macrea et al., 2011; Trayhurn et al., 2008).

DHA application to hypoxic cells increases adiponectin secretion, an observation which is consietnet with recenyt studies (Prostek, Gajewska, Kamola, & Balasinska, 2014; Romacho et al., 2015) and this may refelct the beneficial role of DHA to counteract the stigma of insulin resistance assocaited with hypoxic effcets on adipocytes.

Data attained points towards an increased basal glucose uptake caused by hypoxia through both dependent and independent pathways after the (~24 h)-term hypoxia treatment. Data of the current study demonstrated an increase in the basal glucose uptake and lactate release caused by hypoxia in 3T3-LI adipocytes. Hypoxia increases the glycolytic pathway with increased glucose uptake by several mechanisms including upregulation of certain genes such as hexokinase 2, phopsphofructase (PFKP), and glucose-6 phosphate isomerase (GPI) (Choi et al., 2009). Another mechanism is the upregulation in the expression of glucose transporter GLUT1, which is

responsible for basal glucose uptake as shown in previous studies (Wood, Stezhka, & Trayhurn, 2011). The current data demonstrated marked upregulation of the transcript of GLUT in adipocytes exposed to hypoxia, consistent with previous studies (Trayhurn et al., 2008; Wheeler, Cole, & Hauck, 1998). The increase in GLUT4 expression in the present study could be to secondary upregulation of HIf-1 transcript as shown in a previous study in 3T3-L1 adipocytes with knockout of HIF-1B (Lee et al., 2011). Furthermore, the current study revealed an increased lactate level and a tendency to a decreased pH in the medium of the adipocytes exposed to hypoxia which is consistent with previous results by other investigators (Quintero, Gonzalez-Muniesa, & Martinez, 2012; Wood et al., 2011). Lactate induces the stimulation of inflammation and the induction of insulin resistance in muscles. Insulin resistance associated with hypoxia in adipose tissue may cause the antilipolytic activity with consequently increased lactate production.

Hypoxia regulates the level of mitochondrial production of ATP. With the loss of ATP, it is suspected to be responsible for necrosis and apoptosis. The death of the cells and effect of hypoxia –induced lipolysis leads to a decline in glucose uptake and shift to anaerobic metabolism. Moreover, our data showed a significant decrease of the transcript of the adenine nucleotide translocator (ANT2), which is consistent with a previous study (Chevrollier, Loiseau, Gautier, Malthiery, & Stepien, 2005). ANT2 plays an important role in ATP by maintaining the

mitochondrial integrity and prevent alterations in the proteins involved in oxidative phosphorylation. (Chevrollier et al., 2005).

The current data indicated an increase in the lipolytic activity determined by the release of glycerol and free fatty acids at the basal state determined from the secretions of living adipocytes exposed to the hypoxia. These data are consistent with a previous study showed increased lipolysis in 3T3-L1 adipocytes after hypoxia (1% oxygen) treatment (Yin et al., 2009). The underlying cause of lipolysis could be the impairment action of insulin on adipocytes. Downregulation of PPARγ gene expression was proposed as a mechanism of hypoxia action (K. H. Kim, Song, Chung, Park, & Kim, 2005). Hypoxia was reported to inhibit adipocyte differentiation and adipogenesis (Chen et al., 2006). Our data showed downregulation of PPARγ gene expression that is consistent with the other results (Kim, Song, Chung, Park, & Kim, 2005) and may explain the increased basal lipolysis and decreased adipogenesis after exposure to hypoxia. Moreover, our data showed increased lipid peroxidation (MAD) that is consistent with previous results (Wiernsperger, Nivoit, & Bouskela, 2006). DHA enhance s the basal lipolysis in this study which is consitent with previous studies (H. K. Kim, Della-Fera, Lin, & Baile, 2006; Romacho et al., 2015).

It has been shown that lipolysis in adipocytes may induce the expression of the inflammatory cytokines such as Il-6 and MCP-1, which could suppress the production of adiponectin, which could also be affected by PPAR-y which in turn could lead to insulin resistance and increase of the oxidative stress with increased lipid peroxidation and such effect could be reveresed by DHA treatment as shown in the present stduy (Ashida, Enan, & Matsumura, 1996; Banga et al., 2009; Quintero, Gonzalez-Muniesa, Garcia-Diaz, et al., 2012)

There are two different types of cell deaths that are dependent on ATP, which include the necrosis and apoptosis. Necrosis comes because of complete loss of ATP whereas apoptosis comes as a result of partial loss of ATP. ATP responds to hypoxia by cutting down its production due to the inhibition of respiration functions in the mitochondria. An increase in permeability in the inner mitochondrial membrane is observed in hypoxia (Greijer & van der Wall, 2004).

In summary, the current study shows the effects of hypoxia on 3T3-l1 adipocytes. The exposure to 1% O_2 provokes the release of inflammatory mediators such as Il-6 and MCP-1 with decreased expression of adiponectin, upregulation of HIF-1 and HIf2 and increased basal glucose uptake with upregulation of the GLUT1 and GLUT 4 transcript. Moreover, Hypoxia increased the rate of lipolysis, lactate production, lipid peroxidation, oxidative stress and apoptosis rate. Several genes are altered such as Ant2, PPAR-Y, which are involved with HIF system in such metabolic consequences in response to hypoxia.

Application of DHA to hypoxic cells in the current study counteracts the effects on oxidative stress, apoptosis, and production of pro-inflammatory cytokines, leptin and upregulates adiponectin secretion that could be possible through induction of PPAR gamma (Banga et al., 2009; De Boer et al., 2014; Oster, Tishinsky, Yuan, & Robinson, 2010)

5.2 Limitations and prospective

The study has several limitations, such as use only one level of hypoxia (1% O_2), the continuous nature of the hypoxia treatment, and use of a murine model of adipocytes. Unable to run western blot specially for HIF-1α as confirmatory test due to delayed in rescive shipment on time.

5.3 Future studies

Future studies could be directed to address the limitations discussed above including the use of novel techniques such as next generation sequencing to detect new biomarkers/signaling pathways involved in relevant human tissue from different depots.

5.4 Conclusion

In conclusion, my work demonstrated that adipocytes exposed to hypoxia altered adipokine secretion; the transcript of hypoxia genes and other genes involved in glucose and lipid

metabolism. It is associated with a shift toward the anaerobic metabolism with increased

oxidative stress and apoptosis. In addition the study shows the potent effect of DHA in

counteracting the hypoxic effects on adipocytes and identifies several key factors, which could be

mediating DHA functioning. The beneficial effects of DHA emerged by eliciting a number of

bioactive molecules in 3T3L-1 under hypoxia conditions. These molecules operate within

complex systems such as inflammation factors, lipolysis system, glycolysis process, reactive

oxygen species and various cellular genes involved in adipocytes functioning. The potential of

DHA as a therapeutic agent to reduce side effect of obesity is high as it is readily available, safe

and inexpensive.

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